CHARGE TAGS AND THE SEPARATION OF NUCLEIC ACID MOLECULES

The present invention is a continuation-in-part of pending U.S. patent application No. 09/333,145, which is a continuation application of U.S. Patent No. 6,001,567, herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

The present invention relates to novel phosphoramidites, including positive and neutrally charged compounds. The present invention also provides charge tags for attachment to materials including solid supports and nucleic acids, wherein the charge tags increase or decrease the net charge of the material. The present invention further provides methods for separating and characterizing molecules based on the charge differentials between modified and unmodified materials.

BACKGROUND OF THE INVENTION

Methods for the detection and characterization of specific nucleic acid sequences and sequence variations have been used to detect the presence of viral or bacterial nucleic acid sequences indicative of an infection and to detect the presence of variants or alleles of genes associated with disease and cancers. These methods also find application in the identification of sources of nucleic acids, as for forensic analysis or for paternity determinations. Various methods are known to the art that may be used to detect and characterize specific nucleic acid sequences and sequence variants. Nonetheless, with the completion of the nucleic acid sequencing of the human genome, as well as the genomes of numerous other organisms such as pathogenic organisms, the demand for fast, reliable, cost-effective and user-friendly tests for the detection of specific nucleic acid sequences continues to grow. Importantly, these tests must be able to create a detectable signal from samples that contain very few copies of the sequence of interest.

There are a number of techniques that have been developed for characterizing specific nucleic acid sequences. Examples of detection techniques include the "TaqMan" or nick-translation PCR assay described in U.S. Patent No. 5,210,015 to Gelfand *et al.* (the disclosure of which is herein incorporated by reference), the assays described in U.S.

Patent Nos. 4,775,619 and 5,118,605 to Urdea (the disclosures of which are herein incorporated by reference), the catalytic hybridization amplification assay described in U.S. Patent No. 5,403,711 to Walder and Walder (the disclosure of which is herein incorporated by reference), the cycling probe assay described in U.S. Patents Nos. 4,876,187 and 5,011,769 to Duck *et al.*, the target-catalyzed oligonucleotide modification assay described in U.S. patents 6,110,677 and 6,121,001 to Western *et al.* (the disclosures of which are herein incorporated by reference), the SNP detection methods of Orchid Bioscience in U.S. patent 5,952,174 (the disclosure of which is herein incorporated by reference), the methods of U.S. patent 5,882,867 to Ullman et al. (the disclosure of which is herein incorporated by reference) the polymerase chain reaction (PCR) described in U.S. Patent Nos. 4,683,195, 4,683,202, and 4,965,188 to Mullis and Mullis *et al.* (the disclosures of which are herein incorporated by reference) and the ligase chain reaction (LCR) described in U.S. Patent Nos. 5,427,930 and 5,494,810 to Birkenmeyer *et al.* and Barany *et al.* (the disclosures of which are herein incorporated by reference). The above examples are intended to be illustrative of nucleic acid-based detection assays and do not provide an exhaustive list. Each of these techniques requires a detection step for detecting a reaction product that is indicative of a desired target nucleic acid (e.g., detection of cleavage products, extension products, etc.). While a number of advances have been made in the assay methods and detection instrumentation to improve the sensitively, speed, and cost of detection methods the art is still in need of further improved methods, compositions, and systems to make the assays more sensitive and efficient.

SUMMARY OF THE INVENTION

The present invention relates to novel phosphoramidites, including positive and neutrally charged compounds. The present invention also provides charge tags for attachment to materials including solid supports and nucleic acids, wherein the charge tags increase or decrease the net charge of the material. The present invention further provides methods for separating and characterizing molecules based on the charge differentials between modified and unmodified materials.

For example, the present invention provides a composition comprising a charge tag attached to a nucleic acid molecule (*e.g.*, to a terminal end of a nucleic acid molecule). In some embodiments, the charge tag comprises a phosphate group and a positively charged moiety. In some preferred embodiments, the charge tag further comprises a dye. The present invention is not limited by the position of the individual modular components of the charge tag. For example, in some embodiments, the dye is positioned between the nucleic acid and the positively charged moiety, while in other embodiments, the positively charged moiety is positioned between the nucleic acid and the dye. The present invention is also not limited by the number of each type of component in the charge tag (e.g., the number of dyes, positively charged moieties, etc.). For example, in some embodiments, the charge tag comprises first and second positively charged moieties.

In some embodiments of the present invention, the charge tag has a net positive charge. For example, in some embodiments, the charge tag has a net positive charge of 1, 2, 3, etc. In some embodiments, the charge tag possesses a positive charge only under certain reaction conditions (*e.g.*, pH 6-10).

In some embodiments, the charge tag further comprises one or more nucleotides. In some embodiments, the nucleic acid molecule to which the charge tag is attached contains a sequence that is complementary to a target nucleic acid. In some such embodiments, the one or more nucleotides in the charge are not complementary to the target nucleic acid. In other such embodiments, the nucleic acid comprises a first portion complementary to a target nucleic acid and a second portion that is not complementary to said target nucleic acid, wherein the charge tag is attached to the second portion of the nucleic acid (e.g., to a terminal end of the nucleic acid that is located in the second portion).

In some embodiments of the present invention, the nucleic acid and the charge tag have a combined net neutral charge, wherein the charge tag, in isolation, has a net positive charge. In other embodiments, the nucleic acid and the charge tag have a combined net negative charge, wherein the charge tag has a net positive charge.

The present invention is not limited by the nature of the positively charged moiety of the charge tag. Positively charged moieties include, but are not limited to primary

amines, secondary amines, tertiary amines, ammonium groups, positively charged metal groups (*e.g.*, caged ions attached to the charge tag through a linking group), and the like.

In some embodiments, the charge tag further comprises a positively charged phosphoramidite or a neutral phosphoramidite. The present invention is not limited by the nature of the positively charged phosphoramidite or the neutral phosphoramidite. For example, in some embodiments, the charge tags comprise a novel phosphoramidite of the present invention.

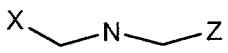
For example, the present invention provides a composition comprising a positively charged phosphoramidite. In some embodiments, the positively charged phosphoramidite contains one or more positively charged moieties including, but not limited to, primary amine groups, secondary amine groups, tertiary amine groups, ammonium groups, charged metal ions, and the like. In some embodiments, the phosphoramidite has a net positive charge of one. In some particularly preferred embodiments, the phosphoramidite has the structure:

wherein, X is a reactive phosphate group (*e.g.*, PO_4) and Y is a protecting group (*e.g.*, dimethoxy trityl [DMT]) and/or a protected group (*e.g.*, DMT-protected hydroxyl group).

The present invention further provides a composition comprising a nucleic acid molecule containing a positively or neutrally charged phosphoramidite. The present invention also provides a composition comprising a charge tag attached to a terminal end of a nucleic acid molecule, wherein the charge tag comprises a positively charged or neutrally charged phosphoramidite. In some preferred embodiments, the positively charged phosphoramite comprises an amine group, wherein the amine group is not further attached to another molecule (a molecule other than the phosphoramidite).

The present invention further provides a composition comprising a neutrally charged phosphoramidite. In some preferred embodiments, the neutrally charged phosphoramidite comprises a nitrogen-containing chemical group selected from the group comprising primary amine, secondary amine, tertiary amine, ammonium group,

and charged metal ion. In some embodiments, the composition further comprises a nucleic acid molecule attached to the neutrally charged phosphoramidite. In some preferred embodiments, the nucleic acid molecule is attached to a charge tag comprising the neutrally charged phosphoramidite. The charge tag may further comprise, in any order, other components. For example, the charge tag may further comprise a positively charged phosphoramidite. In some embodiments of the present invention, the charge tag containing the neutrally charged phosphoramidite has a net positive charge. In some particularly preferred embodiments of the present invention, the neutrally charged phosphoramidite has the structure:



wherein X is a protecting group (*e.g.*, dimethoxy trityl group [DMT]) and/or a protected group (*e.g.*, DMT-protected hydroxyl group), Z is a reactive phosphate, and N comprises an amine group. In some preferred embodiments, the N group is N-$(CH_2)_nCH_3$, wherein n is 0 or a positive integer from 1 to 12.

The present invention also provides a composition comprising a solid support attached to a charge tag. For example, in some embodiments, the charge tag comprises a positively charged moiety and a reactive group configured to allow the charge tag to covalently attach to a nucleic acid molecule. Any of the charge tags described herein, may be attached to the solid support.

The present invention further provides a composition comprising a fluorescent dye directly bonded to a phosphate group, wherein the phosphate group is directly bonded to an amine group. In some embodiments, the composition comprises a charge tag, wherein the fluorescent dye is contained within the charge tag. The present invention is not limited by the nature of the fluorescent dye. However, in some preferred embodiments, the fluorescent dye comprises a Cy dye (*e.g.*, Cy3).

The present invention also provides a mixture comprising a plurality of oligonucleotides attached to charge tags. In some embodiments, each oligonucleotide is attached to a different charge tag. In other embodiments, two or more different oligonucleotides have the same type of charge tag. In some preferred embodiments, each

of the charge tags comprises a phosphate group and a positively charged moiety. While not limited by the number of oligonucleotides attached to different charge tags, in some embodiments, the plurality of oligonucleotides comprises four or more oligonucleotides (*e.g.*, 5, 6, 7, . . ., 10, . . ., 50, . . ., 100, . . .), each attached to a different charge tag. Any of the charge tags described herein are contemplated for use in the mixtures.

The present invention further provides a method of separating nucleic acid molecules, comprising the steps of: a) treating a charge-balanced oligonucleotide containing a charge tag under conditions such that a charge-unbalanced oligonucleotide containing the charge tag is produced, wherein the charge-unbalanced oligonucleotide is contained in a reaction mixture; and b) separating the charge-unbalanced oligonucleotide from the reaction mixture. While the present invention is not limited by the means by which a charge-unbalanced oligonucleotide is generated, in some preferred embodiments, the oligonucleotides are treated with a reactant (*e.g.*, a nuclease). Any of the charge tags described herein are contemplated for use in the method. While the present invention is not limited by the nature of the separation step, contemplated separation steps include, but are not limited to, gel electrophoretic separation, capillary electrophoretic separation, capillary zone electrophoretic separation, and separation is a microchannel.

The present invention also provides a method of separating nucleic acid molecules, comprising the steps of: a) treating a plurality of charge-balanced oligonucleotides, each containing different charge tags, under conditions such that two or more charge-unbalanced oligonucleotides containing the charge tags are produced, wherein the charge-unbalanced oligonucleotides are contained in a reaction mixture; and b) separating the charge-unbalanced oligonucleotides from the reaction mixture. In some preferred embodiments, the separating comprises separating the charge-unbalanced oligonucleotides such that charge-unbalanced oligonucleotides containing different charge tags are separated from one another. Any of the charge tag, oligonucleotide mixtures, and separation methods described herein may be used with this method.

DEFINITIONS

To facilitate an understanding of the present invention, a number of terms and phrases are defined below:

The term "charge-balanced" molecule or oligonucleotide refers to a molecule or oligonucleotide (the input oligonucleotide in a reaction) that has been modified such that the modified molecule or oligonucleotide bears a charge, such that when the modified molecule or oligonucleotide is either reduced in size (*e.g.*, cleaved, shortened, disassociated, unbound, or otherwise altered such that it is part of a complex or molecule having a lower aggregate molecular weight) or increased in sized (*e.g.*, enlarged, elongated, associated, bound, or otherwise altered such that it is part of a complex or molecule having a higher aggregate molecular weight), a resulting product bears a net charge or charge to mass ratio different from the input molecule or oligonucleotide (the resulting molecule thus being a "charge-unbalanced" molecule or oligonucleotide) thereby permitting separation of the input and reacted molecules or oligonucleotides on the basis of charge. The term "charge-balanced" does not imply that the modified or balanced molecule or oligonucleotide has a net neutral charge (although this can be the case). Charge-balancing refers to the design and modification of a molecule or oligonucleotide such that a specific reaction product generated from this input molecule or oligonucleotide can be separated on the basis of charge from the input molecule or oligonucleotide.

For example, in an INVADER oligonucleotide-directed cleavage assay in which the probe oligonucleotide bears the sequence: 5' TTCTTTTCACCAGCGAGACGGG 3' (*i.e.*, SEQ ID NO:1 without the modified bases) and cleavage of the probe occurs between the second and third residues, one possible charge-balanced version of this oligonucleotide would be: 5' Cy3-AminoT-Amino-TCTTTTCACCAGCGAGAC GGG 3' (SEQ ID NO:1). This modified oligonucleotide bears a net negative charge. After cleavage, the following oligonucleotides are generated: 5' Cy3-AminoT-Amino-T 3' and 5' CTTTTCACCAGCGAGACGGG 3' (residues 3-22 of SEQ ID NO:1). 5' Cy3-AminoT-Amino-T 3' bears a detectable moiety (the positively charged Cy3 dye) and two amino-modified bases. The amino-modified bases and the Cy3 dye contribute positive charges in excess of the negative charges contributed by the phosphate groups and thus the 5' Cy3-AminoT-Amino-T 3' oligonucleotide has a net positive charge. The other, longer cleavage fragment, like the input probe, bears a net negative charge. Because the 5' Cy3-AminoT-Amino-T 3' fragment is separable on the basis of charge from the input

probe (the charge-balanced oligonucleotide), it is referred to as a charge-unbalanced oligonucleotide. The longer cleavage products are not generally separated on the basis of charge from the input oligonucleotide as both oligonucleotides bear a net negative charge.

The term "net neutral charge" when used in reference to a molecule or oligonucleotide, including modified oligonucleotides, indicates that the sum of the charges present (*e.g.*, R-NH3+ groups on thymidines, the N3 nitrogen of cytosine, presence or absence or phosphate groups, etc.) under the desired reaction or separation conditions is essentially zero. A molecule or oligonucleotide having a net neutral charge would not migrate in an electrical field.

The term "net positive charge" when used in reference to a molecule or oligonucleotide, including modified oligonucleotides, indicates that the sum of the charges present (*e.g.*, R-NH3+ groups on thymidines, the N3 nitrogen of cytosine, presence or absence or phosphate groups, etc.) under the desired reaction conditions is +1 or greater. A molecule or oligonucleotide having a net positive charge would migrate toward the negative electrode in an electrical field.

The term "net negative charge" when used in reference to a molecule or oligonucleotide, including modified oligonucleotides, indicates that the sum of the charges present (*e.g.*, R-NH3+ groups on thymidines, the N3 nitrogen of cytosine, presence or absence or phosphate groups, etc.) under the desired reaction conditions is -1 or lower. A molecule or oligonucleotide having a net negative charge would migrate toward the positive electrode in an electrical field.

As used herein, the terms "complementary" or "complementarity" are used in reference to polynucleotides (*i.e.*, a sequence of nucleotides such as an oligonucleotide or a target nucleic acid) related by the base-pairing rules. For example, for the sequence " 5'-A-G-T-3'," is complementary to the sequence " 3'-T-C-A-5'." Complementarity may be "partial," in which only some of the nucleic acids' bases are matched according to the base pairing rules. Or, there may be "complete" or "total" complementarity between the nucleic acids. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands. This is of particular importance in amplification reactions, as well as detection

methods which depend upon binding between nucleic acids. Either term may also be used in reference to individual nucleotides, especially within the context of polynucleotides. For example, a particular nucleotide within an oligonucleotide may be noted for its complementarity, or lack thereof, to a nucleotide within another nucleic acid strand, in contrast or comparison to the complementarity between the rest of the oligonucleotide and the nucleic acid strand.

The term "homology" and "homologous" refers to a degree of identity. There may be partial homology or complete homology. A partially homologous sequence is one that is less than 100% identical to another sequence.

As used herein, the term "hybridization" is used in reference to the pairing of complementary nucleic acids. Hybridization and the strength of hybridization (*i.e.*, the strength of the association between the nucleic acids) is influenced by such factors as the degree of complementary between the nucleic acids, stringency of the conditions involved, and the T_m of the formed hybrid. "Hybridization" methods involve the annealing of one nucleic acid to another, complementary nucleic acid, *i.e.*, a nucleic acid having a complementary nucleotide sequence. The ability of two polymers of nucleic acid containing complementary sequences to find each other and anneal through base pairing interaction is a well-recognized phenomenon. The initial observations of the "hybridization" process by Marmur and Lane, Proc. Natl. Acad. Sci. USA 46:453 (1960) and Doty et al., Proc. Natl. Acad. Sci. USA 46:461 (1960) have been followed by the refinement of this process into an essential tool of modern biology.

With regard to complementarity, it is important for some diagnostic applications to determine whether the hybridization represents complete or partial complementarity. For example, where it is desired to detect simply the presence or absence of pathogen DNA (such as from a virus, bacterium, fungi, mycoplasma, protozoan) it is only important that the hybridization method ensures hybridization when the relevant sequence is present; conditions can be selected where both partially complementary probes and completely complementary probes will hybridize. Other diagnostic applications, however, may require that the hybridization method distinguish between partial and complete complementarity. It may be of interest to detect genetic polymorphisms. For example, human hemoglobin is composed, in part, of four

polypeptide chains. Two of these chains are identical chains of 141 amino acids (alpha chains) and two of these chains are identical chains of 146 amino acids (beta chains). The gene encoding the beta chain is known to exhibit polymorphism. The normal allele encodes a beta chain having glutamic acid at the sixth position. The mutant allele encodes a beta chain having valine at the sixth position. This difference in amino acids has a profound (most profound when the individual is homozygous for the mutant allele) physiological impact known clinically as sickle cell anemia. It is well known that the genetic basis of the amino acid change involves a single base difference between the normal allele DNA sequence and the mutant allele DNA sequence.

The complement of a nucleic acid sequence as used herein refers to an oligonucleotide which, when aligned with the nucleic acid sequence such that the 5' end of one sequence is paired with the 3' end of the other, is in "antiparallel association." Certain bases not commonly found in natural nucleic acids may be included in the nucleic acids of the present invention and include, for example, inosine and 7-deazaguanine. Complementarity need not be perfect; stable duplexes may contain mismatched base pairs or unmatched bases. Those skilled in the art of nucleic acid technology can determine duplex stability empirically considering a number of variables including, for example, the length of the oligonucleotide, base composition and sequence of the oligonucleotide, ionic strength and incidence of mismatched base pairs.

As used herein, the term "T_m" is used in reference to the "melting temperature." The melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. Several equations for calculating the T_m of nucleic acids are well known in the art. As indicated by standard references, a simple estimate of the T_m value may be calculated by the equation: $T_m = 81.5 + 0.41(\% G + C)$, when a nucleic acid is in aqueous solution at 1 M NaCl (*see e.g.*, Anderson and Young, Quantitative Filter Hybridization, in Nucleic Acid Hybridization (1985). Other references (*e.g.*, Allawi, H.T. & SantaLucia, J., Jr. Thermodynamics and NMR of internal G.T mismatches in DNA. Biochemistry 36, 10581-94 (1997) include more sophisticated computations which take structural and environmental, as well as sequence characteristics into account for the calculation of T_m.

As used herein the term "stringency" is used in reference to the conditions of temperature, ionic strength, and the presence of other compounds, under which nucleic acid hybridizations are conducted. With "high stringency" conditions, nucleic acid base pairing will occur only between nucleic acid fragments that have a high frequency of complementary base sequences. Thus, conditions of "weak" or "low" stringency are often required when it is desired that nucleic acids which are not completely complementary to one another be hybridized or annealed together.

The term "oligonucleotide" as used herein is defined as a molecule comprising two or more deoxyribonucleotides or ribonucleotides, preferably at least 5 nucleotides, more preferably at least about 10-15 nucleotides and more preferably at least about 15 to 30 nucleotides. The exact size will depend on many factors, which in turn depend on the ultimate function or use of the oligonucleotide. The oligonucleotide may be generated in any manner, including chemical synthesis, DNA replication, reverse transcription, PCR, or a combination thereof.

Because mononucleotides are reacted to make oligonucleotides in a manner such that the 5' phosphate of one mononucleotide pentose ring is attached to the 3' oxygen of its neighbor in one direction via a phosphodiester linkage, an end of an oligonucleotide is referred to as the "5' end" if its 5' phosphate is not linked to the 3' oxygen of a mononucleotide pentose ring and as the "3' end" if its 3' oxygen is not linked to a 5' phosphate of a subsequent mononucleotide pentose ring. As used herein, a nucleic acid sequence, even if internal to a larger oligonucleotide, also may be said to have 5' and 3' ends. A first region along a nucleic acid strand is said to be upstream of another region if the 3' end of the first region is before the 5' end of the second region when moving along a strand of nucleic acid in a 5' to 3' direction.

The term "label" as used herein refers to any atom or molecule that can be used to provide a detectable (preferably quantifiable) signal, and that can be attached to a nucleic acid or protein. Labels may provide signals detectable by fluorescence, radioactivity, colorimetry, gravimetry, X-ray diffraction or absorption, magnetism, enzymatic activity, and the like. A label may be a charged moiety (positive or negative charge) or alternatively, may be charge neutral. Labels can include or consist of nucleic acid or protein sequence, so long as the sequence comprising the label is detectable.

The term "sample" in the present specification and claims is used in its broadest sense. On the one hand it is meant to include a specimen or culture (*e.g.*, microbiological cultures). On the other hand, it is meant to include both biological and environmental samples. A sample may include a specimen of synthetic origin.

Biological samples may be animal, including human, fluid, solid (*e.g.*, stool) or tissue, as well as liquid and solid food and feed products and ingredients such as dairy items, vegetables, meat and meat by-products, and waste. Biological samples may be obtained from all of the various families of domestic animals, as well as feral or wild animals, including, but not limited to, such animals as ungulates, bear, fish, lagamorphs, rodents, etc.

Environmental samples include environmental material such as surface matter, soil, water and industrial samples, as well as samples obtained from food and dairy processing instruments, apparatus, equipment, utensils, disposable and non-disposable items. These examples are not to be construed as limiting the sample types applicable to the present invention.

The term "source of target nucleic acid" refers to any sample that contains nucleic acids (RNA or DNA). Particularly preferred sources of target nucleic acids are biological samples including, but not limited to cultures, blood, saliva, cerebral spinal fluid, pleural fluid, milk, lymph, sputum, semen, and animal or plant tissue.

As used herein, the term "charge tag" refers to a modular chemical complex that is attached to or to be attached to another molecule, wherein the charge tag has a net charge that differs from the net charge of the other molecule. For example, charge tags may be attached to nucleic acid molecules (*e.g.*, to the terminal end of a nucleic acid molecule). Charge tags contain any number of desired components including, but not limited to, dyes, linker groups, nucleotides, phosphoramidites, phosphonates, phosphate groups, amine groups, fluorescent quencher groups and the like.

In a "mixture comprising a plurality of oligonucleotides with each oligonucleotide attached to a different charge tag," two or more oligonucleotides each possess a distinct charge tag, wherein the chemical makeup of the charge tags differ from one another. A mixture of oligonucleotides, each with a different charge tag, may also comprise additional oligonucleotides. For example, the mixture may contain a first set of

oligonucleotides, each with identical first charge tags and a second set of oligonucleotides, each with an identical second charge tags.

As used herein, the term "positively charged moiety" refers to a chemical group or molecule that contains a net positive charge. Positively charged moieties may be attached to or associated with other molecules or materials. A composition containing a positively charged moiety may itself have a net positive charge (because of the positively charged moiety or otherwise), but need not. In some embodiments of the present invention, positively charged moieties include, but are not limited to, amines (*e.g.*, primary, secondary, and tertiary amines). For example, in some embodiments of the present invention, phosphoramidites contain a positively charged moiety comprising an amine. Amine groups are often used as linking chemistries for attaching to or more molecules (*e.g.*, attaching a phosphoramidite to another molecule). However, in some embodiments of the present invention, amine groups are not used as linking groups, but are provided to give a molecule a positive charge. Thus, in some embodiments, the amines are attached to a molecule of interest (*e.g.*, a phosphoramidite), but are not further attached to another molecule (*e.g.*, are not attached to a molecule other than the phosphoramidite).

As used herein, the term "dye" refers to a molecule, compound, or substance that can provide an optically detectable signal (*e.g.*, fluorescent, luminescent, colorimetric, etc). For example, dyes include fluorescent molecules that can be associated with nucleic acid molecules (*e.g.*, Cy3).

As used herein, the term "protecting group" refers to a molecule or chemical group that is covalently attached to a compound to prevent chemical modification of the compound or modification of specific chemical groups of the compound. For example, protecting groups may be attached to a reactive group of a compound to prevent the reactive group from participating in chemical reactions including, for example, intramolecular reactions. In some cases, a protecting group may act as a leaving group, such that when the molecule is added to another compound in a desired synthesis reaction, the protecting group is lost, allowing a reactive group to participate in covalent bonding to the compound. The phosphoramidites of the present invention typically contain one or more protective groups prior to their addition to nucleic acid molecules.

For example, the reactive phosphate of the phosphoramidite (*i.e.*, the phosphate group that is covalently attached to another molecule when the phosphoramidite is added to the other molecule) may contain one or more protecting groups. A detailed description of phosphoramidites and their addition to nucleic acid molecules is provided Beaucage and Iyer (Tetrahedron 49:1925 [1993]), herein incorporated by reference in its entirety.

As used herein, the terms "solid support" or "support" refer to any material that provides a solid or semi-solid structure with which another material can be attached. Such materials include smooth supports (e.g., metal, glass, plastic, silicon, and ceramic surfaces) as well as textured and porous materials. Such materials also include, but are not limited to, gels, rubbers, polymers, and other non-rigid materials. Solid supports need not be flat. Supports include any type of shape including spherical shapes (e.g., beads). Materials attached to solid support may be attached to any portion of the solid support (e.g., may be attached to an interior portion of a porous solid support material). Preferred embodiments of the present invention have biological molecules such as nucleic acid molecules, charge tags, and proteins attached to solid supports. A biological material is "attached" to a solid support when it is associated with the solid support through a non-random chemical or physical interaction. In some preferred embodiments, the attachment is through a covalent bond. However, attachments need not be covalent or permanent. In some embodiments, materials are attached to a solid support through a "spacer molecule" or "linking group." Such spacer molecules are molecules that have a first portion that attaches to the biological material and a second portion that attaches to the solid support. Thus, when attached to the solid support, the spacer molecule separates the solid support and the biological materials, but is attached to both.

As used herein, the term "directly bonded," in reference to two molecules refers to covalent bonding between the two molecules without any intervening linking group or spacer groups that are not part of parent molecules.

As used herein, the terms "linking group" and "linker group" refer to an atom or molecule that links or bonds two entities (*e.g.*, solid supports, oligonucleotides, or other molecules), but that is not a part of either of the individual linked entities.

As used herein, the term "reactant," when referring to an agent that is used to generate charge-unbalanced molecules from charge-balanced molecules, refers to any

agent (*e.g.*, enzyme, chemical, physical device, etc.) that can alter a charge-balanced molecule such that a charge-unbalanced molecule is created.

As used herein, the methods of "capillary electrophoresis," "capillary zone electrophoresis," and "microfluids" refer to methods for use in the separation methods of the present invention. The methods of capillary electrophoresis, capillary zone electrophoresis, and microfluids are described in texts and journals including, but not limited to, Baker (1995) Capillary Electrophoresis, Wiley-Interscience, New York, New York, Weinberger (2000) Capillary Electrophoresis, Second Edition, Academic Press, San Deigo, California, Atamna et al., J. Liq. Chromatogr., 13:2517 (1990), Nishi et al., Anal. Chem., 61:2434 (1989), Terabe et al., Anal. Chem., 56:111 (1984), Bousse et al., Annu. Rev. Biophys. Biomol. Struct., 29:155 (2000), and U.S. Pat. Nos. 5,916,426, 5,807,682, 5,703,222, 5,470,705, 5,777,096, and 5,514,543, each of which is herein incorporated by reference in its entirety.

As used herein, the term "kit" refers to any delivery system for delivering materials. In the context of reaction assays, such delivery systems include systems that allow for the storage, transport, or delivery of reaction reagents (e.g., oligonucleotides, enzymes, etc. in the appropriate containers) and/or supporting materials (e.g., buffers, written instructions for performing the assay etc.) from one location to another. For example, kits include one or more enclosures (e.g., boxes) containing the relevant reaction reagents and/or supporting materials. As used herein, the term "fragmented kit" refers to a delivery systems comprising two or more separate containers that each contain a subportion of the total kit components. The containers may be delivered to the intended recipient together or separately. For example, a first container may contain an enzyme for use in an assay, while a second container contains oligonucleotides. The term "fragmented kit" is intended to encompass kits containing Analyte specific reagents (ASR's) regulated under section 520(e) of the Federal Food, Drug, and Cosmetic Act, but are not limited thereto. Indeed, any delivery system comprising two or more separate containers that each contain a subportion of the total kit components are included in the term "fragmented kit." In contrast, a "combined kit" refers to a delivery system containing all of the components of a reaction assay in a single container (e.g., in a single

box housing each of the desired components). The term "kit" includes both fragmented and combined kits.

DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the chemical structure of several positively charged heterodimeric DNA-binding dyes.

Fig. 2 is the image generated by a fluorescence imager showing thermal degradation of oligonucleotides containing or lacking a 3' phosphate group.

Fig. 3 depicts the structure of amino-modified oligonucleotides 70 and 74.

Fig. 4a depicts the structure of amino-modified oligonucleotide 75

Fig. 4b depicts the structure of amino-modified oligonucleotide 76.

Fig. 5 diagrams the steps leading to the formation of a reactive H-phosphonate intermediate. The wavy lines shown linking the various constituents of these compositions in this and other drawings represent any organic group that can serve this linking purpose.

Fig. 6 diagrams the conversion step leading to the synthesis of **V** and **VI** compounds.

Fig. 7 illustrates the creation of an additional compound **VII** by altering the order of addition of the constituents (compared, *e.g.*, with the order leading to the creation of compound **VI**, Fig. 6).

Fig. 8 illustrates several possible modification configurations for a probe containing two points of modification.

Fig. 9 diagrams the process of introducing a reporter group (*e.g.*, a dye) into a synthesized compound using H-phosphonate chemistry.

Fig. 10 diagrams the release of a positively-charged tag from an oligonucleotide by cleavage in an INVADER assay.

Fig. 11 diagrams five different charge tags, shown as they would be attached to an oligonucleotide.

Fig. 12 diagrams a chiral phosphoramidite.

Fig. 13 diagrams the conversion of a phosphoramidite group to a phosphodiester linkage, as during oligonucleotide synthesis.

Fig. 14 diagrams the general structures of neutral (A) and positively charged (B) phosphoramidites.

Fig. 15 illustrates several possible combinations in the synthesis of a charge balanced probe, using one each of dye, building block, neutral and positively charged phosphoramidites.

Fig. 16 diagrams examples of synthesized neutral and positively charged phosphoramidites.

Fig. 17 shows the structures of a group of charge balances oligonucleotide probes made using neutral and positively charged phosphoramidites.

Fig. 18 is the image generated by a fluorescence imager scan of an IEF gel showing the migration of substrates 70, 70dp, 74, 74dp, 75, 75dp, 76 and 76dp.

Fig. 19A provides a schematic showing an arrangement of a target-specific INVADER oligonucleotide (SEQ ID NO:2) and a target-specific probe oligonucleotide (SEQ ID NO:11) bearing a 5' Cy3 label along a target nucleic acid (SEQ ID NO:49).

Fig. 19B is the image generated by a fluorescence imager showing the detection of specific cleavage products generated in an invasive cleavage assay using charge reversal (*i.e.*, charge based separation of cleavage products).

Fig. 20 is the image generated by a fluorescence imager that depicts the sensitivity of detection of specific cleavage products generated in an invasive cleavage assay using charge reversal.

Figs. 21A and 21B are images generated by a fluorescence imager showing the products produced using the CLEAVASE A/G and *Pfu* FEN-1 nucleases and probes having or lacking a 5' positive charge; the gel shown in Fig. 21A was run in the standard direction and the gel shown in Fig. 21B was run in the reverse direction.

Fig. 22 shows a graph comparing rates of cleavage of charge-modified probes.

Fig. 23A shows a schematic diagram of an H-phosphonate (HP)-charge modified probe in an invasive cleavage.

Fig. 23B diagrams the structures of the charge-modified nucleoside (dN) and hexanol (HEX) tags.

Fig. 24A is an image generated by a fluorescence imager showing the products of cleavage of 5 different charge-balanced probes, resolved by gel electrophoresis run in the standard direction.

Fig. 24B is an image generated by a fluorescence imager showing the products of cleavage of 5 different charge-balanced probes, resolved by gel electrophoresis run in the reverse direction.

Fig. 25 shows a graph comparing the rates of cleavage of five charge balanced probes and one fluorescein-labeled control probe.

Fig. 26A shows a graph comparing the rates of specific signal accumulation in reaction performed for different times, ranging from one to twenty four hours.

Fig 26B shows a graph comparing the amounts of background signal detected in reactions performed for different times, ranging from one to twenty four hours.

Fig. 27 is an image generated by a fluorescence imager showing the products of cleavage of four different charge-balanced probes, either alone or combined in a single lane, resolved by gel electrophoresis run in the reverse direction.

Fig. 28A shows a schematic diagram of oligonucleotides used for the detection of human MCP-1 RNA in a cascading cleavage reaction releasing a charge tag for detection.

Fig. 28B shows a schematic diagram of oligonucleotides used for the detection of human Ubiquitin RNA in a cascading cleavage reaction releasing a charge tag for detection.

Fig. 29 is an image generated by a fluorescence imager showing the products of INVADER assays for the detection of human MCP-1 and ubiquitin mRNAs alone or combined in the same reaction. Products were resolved by gel electrophoresis run in the reverse direction.

Fig. 30A shows images generated by a fluorescence imager, comparing the products of INVADER assays for the detection of human MCP-1 and ubiquitin RNAs either alone or combined in the same reaction, and resolved by gel electrophoresis run in either the reverse or normal polarity.

Fig. 30B shows images generated by a fluorescence imager, comparing the products of INVADER assays for the detection of human MCP-1 and ubiquitin RNAs

either alone or combined in the same reaction, and resolved by gel electrophoresis run in either the reverse or normal polarity.

Fig. 31 shows micellar electrokinetic chromatography (MECC) profiles showing the effects of sample buffer components on CE resolution.

Fig. 32 shows MECC profiles showing the effects of injection time on CE resolution.

Fig. 33 shows MECC profiles showing the effects of capillary type on CE resolution.

Fig. 34 shows MECC profiles showing the effects of ionic strength of the separation buffer on CE resolution.

Fig. 35 shows MECC profiles showing the effects of the pH of the separation buffer on CE resolution.

Fig. 36 shows MECC profiles showing the effects of the concentration of Bis-Tris borate buffer on CE resolution.

Fig. 37 shows MECC profiles showing the effects of the detergent of the efficiency of CE resolution.

Fig. 38 shows MECC profiles for the four net positively charged tags, 5'-V-Cy3-C-3', 5'-V-(dA)-Cy3-C-3', 5'-V-(dG)-Cy3-C-3', and 5'-V-(dT)-Cy3-C-3', separated individually and as an equimolar mixture of all four molecules.

Fig. 39 shows MECC profiles demonstrating the effect of the use of a fresh capillary on the separation of the tag mixture shown in Fig. 38.

Fig. 40 shows MECC profiles for each of six net positively charged tags separated individually or as an equimolar mixture of all six molecules.

Fig. 41 shows images generated by a fluorescence imager comparing the mobility of 5'-Tag1-G-3' or 5'-Tag2-G-3' under the conditions of a denaturing gel (A) to the mobility under conditions of a native gel (B).

DESCRIPTION OF THE INVENTION

As described above, some nucleic acid-based detection assays involve the elongation and/or shortening of oligonucleotide probes. For example, as described herein, the primer-directed, primer-independent, and INVADER-directed cleavage

assays, as well as the "nibbling" assay all involve the cleavage (*i.e.*, shortening) of oligonucleotides as a means for detecting the presence of a target nucleic sequence. Examples of other detection assays that involve the shortening of an oligonucleotide probe include the "TaqMan" or nick-translation PCR assay, the assays described in U.S. Patent Nos. 4,775,619 and 5,118,605 to Urdea, the catalytic hybridization amplification assay described in of Walder and Walder, the cycling probe assay of Duck *et al.*, and the target-catalyzed oligonucleotide modification assay of Western. Examples of detection assays that involve the elongation of an oligonucleotide probe (or primer) include the SNP detection methods of Orchid Bioscience in U.S. patent 5,952,174, the methods of U.S. patent 5,882,867 to Ullman et al., the polymerase chain reaction (PCR), and the ligase chain reaction (LCR). The above examples are intended to be illustrative of nucleic acid-based detection assays that involve the elongation and/or shortening of oligonucleotide probes and do not provide an exhaustive list.

Typically, nucleic acid-based detection assays that involve the elongation and/or shortening of oligonucleotide probes require post-reaction analysis to detect the products of the reaction. It is common that the specific reaction product(s) must be separated from the other reaction components, including the input or unreacted oligonucleotide probe. One detection technique involves the electrophoretic separation of reacted and unreacted oligonucleotide probes. When the assay involves the cleavage or shortening of a probe, the unreacted product will be longer than the reacted or cleaved product. When the assay involves the elongation of a probe (or primer), the reaction products will be greater in length than the unreacted probes. Gel-based electrophoresis of a sample containing nucleic acid molecules of different lengths separates these fragments primarily on the basis of size. This is due to the fact that, in solutions having a neutral or alkaline pH, nucleic acids having widely different sizes (*i.e.*, molecular weights) possess very similar charge-to-mass ratios and do not separate based solely on charge (Andrews, Electrophoresis, 2nd Edition, Oxford University Press (1986), pp. 153-154). The gel matrix acts as a molecular sieve and allows nucleic acids to be separated on the basis of size and shape (*e.g.*, linear, relaxed circular or covalently closed supercoiled circles). Unmodified nucleic acids have a net negative charge due to the presence of negatively charged phosphate groups contained within the sugar-phosphate backbone of the nucleic

acid. Typically, the sample is applied to gel near the negative pole and the nucleic acid fragments migrate into the gel toward the positive pole with the smallest fragments moving fastest through the gel. For gel electrophoresis to effectively resolve different fragments (i.e., to make them distinguishable from each other), the differences in size or shape must be great enough to cause perceptible differences in the rates of migration of the different fragments through the gel.

The present invention provides novel compositions and methods for characterizing molecules, including nucleic acid molecules, based on differences in charge between starting molecules and molecules that have undergone a modification to add or remove one or more chemical constituents. For example, the present invention provides novel methods and compositions for modifying nucleic acid molecules wherein a cleaved or elongated nucleic acid molecule contains a different charge than unmodified nucleic acids, allowing for the efficient separation and detection of the reacted molecules. While the charge-based separation methods of the present invention are applicable to any number of systems (e.g., separation and characterization of products and intermediates in chemical synthesis and drug design) , and are not limited to the use of nucleic acids, the following description focuses on nucleic acid applications to illustrate certain preferred aspects of the present invention.

The detailed description of the invention is presented in the following sections:

I. Fractionation Of Specific Nucleic Acids By Selective Charge Reversal

a. Applications in INVADER assay cleavage reactions

II. Positively Charged Moieties in the Synthesis of Charge-Balanced Molecules

a. H-phosphonate Chemistry

b. A New Class of Phosphoramidite Building Blocks

I. Fractionation Of Specific Nucleic Acids By Selective Charge Reversal

The present invention provides a novel means for fractionating nucleic acid fragments on the basis of charge. This novel separation technique is related to the observation that positively charged adducts can affect the electrophoretic behavior of small oligonucleotides because the charge of the adduct is significant relative to charge of the whole complex. In addition to the use of positively charged adducts (*e.g.*, Cy3 and

Cy5 fluorescent dyes, the positively charged heterodimeric DNA-binding dyes shown in Fig. 1, etc.), the oligonucleotide may contain amino acids (particularly useful amino acids are the charged amino acids: lysine, arginine, asparate, glutamate), polypeptides, modified bases, such as amino-modified bases, charged ions or metals, a phosphonate backbone (at all or a subset of the positions), or any other chemical or molecular constituent that adds to the net positive charge of the oligonucleotide. In other embodiments, as discussed further below, a neutral dye or detection moiety (*e.g.*, biotin, streptavidin, etc.) may be employed in place of a positively charged adduct, in conjunction with the use of amino-modified bases and/or a complete or partial phosphonate backbone.

This observed effect is of particular utility in assays based on the cleavage of DNA molecules. Using the INVADER assays described herein as an example, when an oligonucleotide is shortened through the action of a CLEAVASE enzyme or other cleavage agent, the positive charge can be made to not only significantly reduce the net negative charge, but to actually override it, effectively "flipping" the net charge of the labeled entity. This reversal of charge allows the products of target-specific cleavage to be partitioned from uncleaved probe by extremely simple means. For example, the products of cleavage can be made to migrate towards a negative electrode placed at any point in a reaction vessel, for focused detection without gel-based electrophoresis. When a slab gel is used, sample wells can be positioned in the center of the gel, so that the cleaved and uncleaved probes can be observed to migrate in opposite directions. Alternatively, a traditional vertical gel can be used, but with the electrodes reversed relative to usual DNA gels (*i.e.*, the positive electrode at the top and the negative electrode at the bottom) so that the cleaved molecules enter the gel, while the uncleaved disperse into the upper reservoir of electrophoresis buffer. Similarly, the electrodes of a capillary or microchannel device can be configured so that positively charged cleaved molecules preferentially enter the capillary or channel for separation.

An significant benefit of this type of readout is the absolute nature of the partition of products from substrates (*i.e.*, the separation may be as high as 100%). This means that an abundance of uncleaved probe can be supplied to drive the hybridization step of a probe-based assay, yet the unconsumed (*i.e.*, unreacted) probe can, in essence, be

subtracted from the result to reduce background by virtue of the fact that the unreacted probe will not migrate toward the same pole as the specific reaction product.

Through the use of multiple positively charged adducts, synthetic molecules can be constructed with sufficient modification that the normally negatively charged strand is made nearly neutral. When so constructed, the presence or absence of a single phosphate group can mean the difference between a net negative or a net positive charge. This observation has particular utility when one objective is to discriminate between enzymatically generated fragments of DNA, which generally lack a 3' phosphate, and the products of thermal degradation, which generally retain a 3' phosphate (and thus two additional negative charges, Fig. 2). Examples 1 and 2 demonstrate the ability to separate positively charged reaction products from a net negatively charged substrate oligonucleotide. As discussed in these examples, oligonucleotides may be transformed from net negative to net positively charged compounds. In Example 2, the positively charged dye, Cy3 was incorporated at the 5' end of a 22-mer (SEQ ID NO:1) which also contained two amino-substituted residues at the 5' end of the oligonucleotide; this oligonucleotide probe carries a net negative charge. After cleavage, which occurred 2 nucleotides into the probe, the following labeled oligonucleotide was released: 5'-Cy3-AminoT-AminoT-3' (in addition to unlabeled fragment comprising the remaining 20 nucleotides of SEQ ID NO:1). This short fragment bears a net positive charge while the remainder of the cleaved oligonucleotide and the unreacted or input oligonucleotide bear net negative charges.

The present invention contemplates embodiments wherein the specific reaction product produced by any cleavage of any oligonucleotide or molecule can be designed to carry a net positive charge while the unreacted molecule is charge neutral or carries a net negative charge. The present invention also contemplates embodiments where the released product may be designed to carry a net negative charge while the input nucleic acid carries a net positive charge. Depending on the length of the released product to be detected, positively charged dyes may be incorporated at the one end of the probe and modified bases may be placed along the oligonucleotide such that upon cleavage, the released fragment containing the positively charged dye carries a net positive charge. Amino-modified bases may be used to balance the charge of the released fragment in

cases where the presence of the positively charged adduct (*e.g.*, dye) alone is not sufficient to impart a net positive charge on the released fragment. In addition, the phosphate backbone may be replaced with a phosphonate backbone at a level sufficient to impart a net positive charge (this is particularly useful when the sequence of the oligonucleotide is not amenable to the use of amino-substituted bases); Figs. 3 and 4 show the structure of short oligonucleotides containing a phosphonate group on the second T residue). An oligonucleotide containing a fully phosphonate-substituted backbone would be charge neutral (absent the presence of modified charged residues bearing a charge or the presence of a charged adduct) due to the absence of the negatively charged phosphate groups. Phosphonate-containing nucleotides (*e.g.*, methylphosphonate-containing nucleotides) are readily available and can be incorporated at any position of an oligonucleotide during synthesis using techniques that are well known in the art.

In essence, in these embodiments the invention contemplates the use of charge-based separation to permit the separation of specific reaction products from the input oligonucleotides in nucleic acid-based detection assays. The foundation of this novel separation technique is the design and use of oligonucleotide probes (typically termed "primers" in the case of PCR) that are "charge balanced" so that upon either cleavage or elongation of the probe it becomes "charge unbalanced," and the specific reaction products may be separated from the input reactants on the basis of the net charge.

In some embodiments, in the context of assays that involve the elongation of an oligonucleotide probe (*i.e.*, a primer), such as is the case in PCR, the input primers are designed to carry a net positive charge. Elongation of the short oligonucleotide primer during polymerization will generate PCR products that now carry a net negative charge. The specific reaction products may then easily be separated and concentrated away from the input primers using the charge-based separation technique described herein.

a. Applications in INVADER assay cleavage reactions

i. Detection of Specific Nucleic Acid Sequences Using 5' Nucleases in an INVADER Directed Cleavage Assay

The present invention finds application in the detection of cleavage products generated in the INVADER assay. The INVADER assay provides means for forming a nucleic acid cleavage structure that is dependent upon the presence of a target nucleic acid and cleaving the nucleic acid cleavage structure so as to release distinctive cleavage products. 5' nuclease activity, for example, is used to cleave the target-dependent cleavage structure and the resulting cleavage products are indicative of the presence of specific target nucleic acid sequences in the sample. When two strands of nucleic acid, or oligonucleotides, both hybridize to a target nucleic acid strand such that they form an overlapping invasive cleavage structure, as described below, invasive cleavage can occur. Through the interaction of a cleavage agent (e.g., a 5' nuclease) and the upstream oligonucleotide, the cleavage agent can be made to cleave the downstream oligonucleotide at an internal site in such a way that a distinctive fragment is produced. Such embodiments have been termed the INVADER assay (Third Wave Technologies) and are described in U.S. Patent Nos. 5,846,717, 5,985,557, 5,994,069, 6,001,567, and 6,090,543 and PCT Publications WO 97/27214 and WO 98/42873, herein incorporated by reference in their entireties.

The INVADER assay further provides assays in which the target nucleic acid is reused or recycled during multiple rounds of hybridization with oligonucleotide probes and cleavage of the probes without the need to use temperature cycling (i.e., for periodic denaturation of target nucleic acid strands) or nucleic acid synthesis (i.e., for the polymerization-based displacement of target or probe nucleic acid strands). When a cleavage reaction is run under conditions in which the probes are continuously replaced on the target strand (*e.g.* through probe-probe displacement or through an equilibrium between probe/target association and disassociation, or through a combination comprising these mechanisms, (Reynaldo *et al.*, J. Mol. Biol. 97:511 [2000])), multiple probes can hybridize in turn to the same target, allowing multiple cleavages, and the generation of multiple cleavage products.

By the extent of its complementarity to a target nucleic acid strand, an oligonucleotide may be said to define a specific region of said target. In an invasive cleavage structure, the two oligonucleotides define and hybridize to regions of the target that are adjacent to one another (*i.e.*, regions without any additional region of the target between them). Either or both oligonucleotides may comprise additional portions that are not complementary to the target strand. In addition to hybridizing adjacently, in order to form an invasive cleavage structure, the 3' end of the upstream oligonucleotide must comprise an additional moiety. When both oligonucleotides are hybridized to a target strand to form a structure and such a 3' moiety is present on the upstream oligonucleotide within the structure, the oligonucleotides may be said to overlap, and the structure may be described as an overlapping, or invasive cleavage structure.

In one embodiment, the 3' moiety of the invasive cleavage structure is a single nucleotide. In this embodiment the 3' moiety may be any nucleotide (*i.e.*, it may be, but it need not be complementary to the target strand). In a preferred embodiment, the 3' moiety is a single nucleotide that is not complementary to the target strand. In another embodiment, the 3' moiety is a nucleotide-like compound (*i.e.*, a moiety having chemical features similar to a nucleotide, such as a nucleotide analog or an organic ring compound; *See e.g.*, U.S. Pat. No. 5,985,557). In yet another embodiment the 3' moiety is one or more nucleotides that duplicate in sequence one or more nucleotides present at the 5' end of the hybridized region of the downstream oligonucleotide. In a further embodiment, the duplicated sequence of nucleotides of the 3' moiety is followed by a single nucleotide that is not further duplicative of the downstream oligonucleotide sequence, and that may be any other nucleotide. In yet another embodiment, the duplicated sequence of nucleotides of the 3' moiety is followed by a nucleotide-like compound, as described above.

The downstream oligonucleotide may have additional moieties attached to either end of the region that hybridizes to the target nucleic acid strand. In a preferred embodiment, the additional moiety comprises a tag of the present invention. In a particularly preferred embodiment, the downstream oligonucleotide comprises a tag or other moiety at its 5' end (*i.e.*, a 5' moiety).

When an overlapping cleavage structure is formed, it can be recognized and cleaved by a nuclease that is specific for this structure (*i.e.*, a nuclease that will cleave one or more of the nucleic acids in the overlapping structure based on recognition of this structure, rather than on recognition of a nucleotide sequence of any of the nucleic acids forming the structure). Such a nuclease may be termed a "structure-specific nuclease." In some embodiments, the structure-specific nuclease is a 5' nuclease. In a preferred embodiment, the structure-specific nuclease is the 5' nuclease of a DNA polymerase. In another preferred embodiment, the DNA polymerase having the 5' nuclease is synthesis-deficient. In another preferred embodiment, the 5' nuclease is a FEN-1 endonuclease. In a particularly preferred embodiment, the 5' nuclease is thermostable.

In some embodiments, the structure-specific nuclease preferentially cleaves the downstream oligonucleotide. In a preferred embodiment, the downstream oligonucleotide is cleaved one nucleotide into the 5' end of the region that is hybridized to the target within the overlapping structure. Cleavage of the overlapping structure at any location by a structure-specific nuclease produces one or more released portions or fragments of nucleic acid, termed "cleavage products."

Detection of the cleavage products may be through release of a label. Such labels may include, but are not limited to one or more of any of dyes, radiolabels such as ^{32}P or ^{35}S, binding moieties such as biotin, mass tags, such as metal ions or chemical groups, charge tags, such as polyamines or charged dyes, haptens such as digoxgenin, luminogenic, phosphorescent or fluorogenic moieties, and fluorescent dyes, either alone or in combination with moieties that can suppress or shift emission spectra, such as by fluorescence resonance energy transfer (FRET) or collisional fluorescence energy transfer.

Examples 1-3 and 9-18, below, demonstrate the use of charge balanced oligonucleotides in the INVADER assay. Cleavage results in the production of charge unbalanced products which are readily separated from the input molecules. The cleavage products are easily detected, providing an efficient and sensitive assay.

II. Positively Charged Moieties in the Synthesis of Charge-Balanced DNA Probes

The present invention provides novel positively charged moieties that may be attached to any number of molecules, including nucleic acid molecules. These positively charged moieties find use in the charge reversal separation methods ("CRE" methods) of the present invention. As used herein, the term "positively charged moiety" refers to a chemical structure that possesses a net positive charge under the reaction conditions of its intended use (e.g., when attached to a molecule of interest under the pH of the desired reaction conditions). Positively charged moieties need not always carry a positive charge. Indeed, in some preferred embodiments of the present invention, the positively charged moiety does not carry a positive charge until it is introduced to the appropriate reaction conditions. This can also be thought of as "pH-dependent" and "pH-independent" positive charges. pH-dependent charges are those that possess the charge only under certain pH conditions, while pH-independent charges are those that possess a charge regardless of the pH conditions.

The positively charged moieties, or "charge tags," when attached to another entity, can be represented by the formula:

X-Y

where X is the entity (e.g., a solid support, a nucleic acid molecule, etc.) and Y is the charge tag. The charge tags can be attached to other entities through any suitable means (e.g., covalent bonds, ionic interactions, etc.) either directly or through an intermediate (e.g., through a linking group). In preferred embodiments, where X is a nucleic acid molecule, the charge tag is attached to either the 3' or 5' end of the nucleic acid molecule.

The charge tags may contain a variety of components. For example, the charge tag Y can be represented by the formula:

Y_1-Y_2

where Y_1 comprises a chemical component that provides the positive charge to the charge tag and where Y_2 is another desired component. Y_2 may be, for example, a dye, another chemical component that provides a positive charge to the charge tag, a functional group for attachment of other molecules to the charge tag, a nucleotide, etc. Where such a structure is attached to another entity, X, either Y_1 or Y_2 may be attached to X.

$X\text{-}Y_1\text{-}Y_2$ or $X\text{-}Y_2\text{-}Y_1$.

The charge tags are not limited to two components. Charge tags may comprise any number of desired components. For example, the charge tag can be represented by the formula:

$Y_1\text{-}Y_2\text{-}Y_3\text{-}Y_n$ (n = any positive integer).

where any of the Y groups comprises a chemical component that provides the positive charge to the charge tag and where the other Y groups are any other desired components. For example, in some embodiments, the present invention provides compositions of the structure:

$X\text{-}Y_1\text{-}Y_2\text{-}Y_3\text{-}Y_4$

where X is an entity attached to the charge tag (e.g., a solid support, a nucleic acid molecule, etc.) and where Y_1 is a dye, Y_2 is a chemical component that provides the positive charge to the charge, Y_3 is a component containing a functional group that allows the attachment of other molecules, and Y_4 is a second chemical component that provides a positive charge. The identity of each of Y_1-Y_4 can be interchanged (i.e., the present invention is not limited by the order of the components).

The present invention is not limited by the nature of the chemical components that provides the positive charge to the charge tag. Such chemical components include, but are not limited to, amines (primary, secondary, and tertiary amines), ammoniums, and

phosphoniums. The chemical components may also comprise chemical complexes that entrap or are otherwise associated with one or more positively charged metal ions.

In preferred embodiments of the present invention, charge tags are attached to nucleic acid molecules (e.g., DNA molecules). The charge tags may be synthesized directly onto a nucleic molecule or may be synthesized, for example, on a solid support or in liquid phase and then attached to a nucleic acid molecule or any other desired molecule. In some preferred embodiments of the present invention, charge tags that are attached to nucleic acid molecules comprise one or more components synthesized by H-phosphonate chemistry (described in detail below), by incorporation of novel phosphoramidites (described in detail below), or a combination of both. For example, compositions of the present invention include structures such as:

$[X]-[Y_1-Y_2-Y_3-Y_4]$

where [X] is a nucleic acid molecule and [Y . . .] is a charge tag. In some embodiments, Y_1 is a dye, Y_2 is synthesized using H-phosphonate chemistry and comprises a chemical component that provides a positive charge to the charge tag, Y_3 is a positively charged phosphoramidite, and Y_4 is a nucleotide or polynucleotide. Any of the Y components are interchangeable with one another.

Such compositions find use in the charge-separation assays of the present invention. For example, a probe molecule in the INVADER assay may have a charge tag attached to its 5' end. The probe may comprise a net negative charge because of the plurality of negatively charge phosphate groups in the oligonucleotide backbone. Cleavage of the probe releases the charge tag from the rest of the probe. The released cleavage fragment, containing the charge tag, carries a net positive charge, while the remaining probe oligonucleotide carries a net negative charge. The cleaved fragments can then be readily separated from the uncleaved probes and detected, indicating the presence of a specific target sequence in the experimental sample.

a. H-Phosphonate chemistry.

As discussed above, one or more components of a charge tag can be synthesized using H-phosphonate chemistry. Production of charge tags using the methods described herein provides a convenient and flexible modular approach for the design of a wide variety of charge tags. Since its introduction, solid phase H-phosphonate chemistry (B.C. Froehler, Methods in Molecular Biology, 20:33, S. Agrawal, Ed. Humana Press; Totowa, New Jersey[1993]) has been recognized as an efficient tool in the chemical synthesis of natural, modified and labeled oligonucleotides and DNA probes. Those skilled in the art know that this approach allows for the synthesis of the oligonucleotide fragments with a fully modified phosphodiester backbone (*e.g.,* oligonucleotide phosphorothioates; Froechler [1993], *supra*) or the synthesis of oligonucleotide fragments in which only specific positions of the phosphodiester backbone are modified (Agrawal, *et al.*, Proc.Natl.Acad.Sci USA , 85:7079 [1988], Froehler,Tetrahedron Lett. 27:5575[1986], Froehler, *et al.*, Nucl.Acids Res. 16:4831 [1988]). The use of H-phosphonate chemistry allows for the introduction of different types of modifications into the oligonucleotide molecule (Agrawal, *et al.,* Froehler[1986], *supra*, Letsinger, *et al.*, J.Am. Chem.Soc., 110:4470 [1988], Agrawal and Zamecnik, Nucl. Acid Res. 18:5419 [1990], Handong, *et al.*, Bioconjugate Chem. 8:49 [1997], Vinogradov, *et al.*, Bioconjugate Chem. 7:3 [1995], Schultz, *et al.*, Tetrachedron Lett. 36:8407 [1995]), however the replacement of the phosphodiester linkage by the phosphoramidate linkage is one of the most frequent changes due to its effectiveness and synthetic flexibility. Froehler and Letsinger were among first to use this approach in the synthesis of modified oligonucleotides in which phosphodiester linkages were fully or partially replaced by the phosphoramidate linkages bearing positively charged groups (e.g., tertiary amino groups; Froehler [1986], Froehler, *et al.,* [1988], and Letsinger, *et al.*, *supra*).

In some embodiments of the present invention, charge tags are generated using H-phosphonate chemistry. The charge tags may be assembled on the end of a nucleic acid molecule or may be synthesized separately and attached to a nucleic acid molecule. Any suitable phosphorylating agent may be used in the synthesis of the charge tag. For example, the component to be added may contain the structure:

where A is a protecting group, B is any desired functional group (e.g., a functional group that provides a positive charge to the charge tag), and P is a chemical group containing phosphorous. In preferred embodiments, B comprises a chemical group that is capable of providing a positive charge to the charge tag. However, in some embodiments B is a functional group that allows post-synthetic attachment of a positively charged group to the charge tag.

The process of the synthesis of the charge-balanced charge tag containing (CRE) probes using H-phosphonate chemistry can be divided into steps.

1. In the first step, the specific DNA sequence is synthesized using a standard automated phosphoramidite protocol (a reporter molecule (dye) may be introduced into the molecule at this stage using phosphoramidite or H-phosphonate chemistry, or it can be attached to the probe after the completion of other steps of the modification procedure using any of the standard post-synthetic labeling protocols).

2. In the second step, a modification procedure is performed using solid-phase H-phosphonate chemistry. The DNA probe, suspended on the solid support, is coupled to an appropriate H-phosphonate monomer in the presence of an appropriate activating reagent (e.g., pivaloyl chloride). This step leads to the formation of the reactive H-phosphonate intermediate (Fig. 5).

Group "Z" in Fig. 5 represents any organic group (with any other functional groups present protected as necessary for protocols of chemical synthesis of oligonucleotides). Group "Z" may optionally contain other DMT-protected hydroxyl groups (or other appropriately protected functional groups), to which additional monomeric units (*e.g.*, H-phosphonate, phosphoramidite, etc.) can be attached, either covalently or noncovalently (e.g. thorough complex formation). Wavy lines in Fig. 5 and other figures in this patent disclosure, *e.g.*, as shown linking controlled pore glass (CPG) and the DNA molecule (and which may link any two entities of these compositions), represent any kind of atom or organic group that can serve these purposes.

This step should be performed on a DNA synthesizer with H-phosphonate adaptation or should be performed manually according to a solid phase H-phosphonate coupling protocol.

A subsequent step of the modification procedure involves the conversion of the intermediate H-phosphonate into the phosphoramidate-bearing group(s) that can introduce positive charges into the composition. Usually, this conversion is performed with the help of an Atherton-Tod reaction, in which the intermediate H-phosphonate III or IV is treated with a solution of an appropriate primary or secondary amine, carbon tetrachloride (or other reagent(s) leading to the same type of transformation in which phosphoramidate bond between the amine used in the reaction and phosphorus atom is formed) in anhydrous aprotic solvent(s), preferably pyridine, mixture of pyridine and acetonitrile, or pyridine and tetrahydrofuran. Fig. 6 shows the conversion leading to the synthesis of V and VI.

The structure of the monomeric H-phosphonate may optionally contain additional, appropriately protected functional groups (*e.g.*, amino, hydroxyl, mercapto or carboxy groups) that can be used in other steps of the synthesis and modification of the probe containing the charge tag.

If the modification procedure involves multiple coupling steps performed using H-phosphonate chemistry or phosphoramidite chemistry, the H-phosphonate monomer(s) used in the modification procedure should contain selectively protected hydroxyl group, preferably with the DMT protecting group, while other functional groups should be protected with protecting groups compatible with the protocol of the chemical synthesis of oligonucleotides.

It is important to note that the possibility of the use of the intermediate materials I or II significantly increases the synthetic flexibility of the modification procedure (and helps to create a broad variety of charge-balanced probes). By altering the sequence of coupling of the H-phosphonate reagents and another reagents (e.g. reporter molecules) to the synthesized DNA sequence, different probes (CRE-VI) can be synthesized. The probes generate fragments of varying polarity and/or mobility upon cleavage in, for example, an INVADER assay. The synthetic flexibility of the H-phosphonate approach

can be conveniently illustrated on the example of the synthesis of the multiple labeled CRE probe.

Introduction of multiple points of modification with moieties bearing positive charge(s) may be desired, in order to compensate negative charges introduced into the probe by another group (*e.g.*, a dye bearing multiple negative charges or other groups).

The synthesis of CRE probes containing only two points of modification, one introducing a positively charged moiety and one introducing a neutral group for structure modulation, and having only one dye that does not alter the net charge (*e.g.*, Cy3 dye introduced using phosphoramidite chemistry), is illustrated in Fig. 8.

As it can be seen, the synthetic procedure in which only one reporter group, one type of H-phosphonate monomer and two different amines were used, can generate six different charge-balanced CRE probes. The number of possible structural variations of the synthesized charge-balanced CRE probes using a single reporter molecule (*e.g,*. Cy3) can be significantly expanded if the synthesis is performed using one of two structurally different H-phosphonate monomers, one of two different amines for introducing positive charge, and one of two different amines for structure modulation. The use of those reagents will lead to the creation of four different modifications introducing positive charge and four different structure modulating modifications.

In the discussed example, the structure of a charge-balanced CRE probe should contain one position occupied by a reporter molecule (*e.g.* Cy3), one position occupied by a modification introducing positive charge and (optionally) one position occupied by a structure-modulating modification. A total 96 different charge-balanced CRE probes can be synthesized using the above mentioned reagents.

It is clear that a large number of possible structural permutations are achieved with the use of only seven different reagents, allowing for the selection of the structural arrangement that will offer a particular desired probe performance (*i.e.*, assay performance and/or the desired electrophoretic mobility of the cleaved positively charged fragments). The same set of reagents can be used in the synthesis of charge balanced probes that do not contain any neutral modifications (*e.g.*, as used for structure modulation) or that contain multiple points at which structure-modulating modification can be added. This further expands the number of possible structures of charge-balanced

probes that can be synthesized using a relatively small (seven in the discussed example) number of reagents. It is important to note that reporter groups can be also introduced into CRE probes using H-phosphonate chemistry. Fig. 9 diagrams a process in which an activated H-phosphonate of a reporter molecule (e.g., a dye) reacts with an available hydroxyl group of an oligonucleotide attached to a solid phase, leading to the formation of an intermediate H-phosphonate IVa, which is subsequently converted to a phosphoramidate-derivative using an appropriate primary or secondary amine and the chemical reaction described above.

In all cases, these procedures lead to the attachment of a specific structure of charged organic moiety (described later as $COM^{(+)}$) to a DNA sequence. As a result, a positively charged fragment (positively charged Tag; called later "PCT") cleaved in the enzymatic process, will be composed of one nucleotide and the $COM^{(+)}$, and will have the desired net positive charge (Fig. 10).

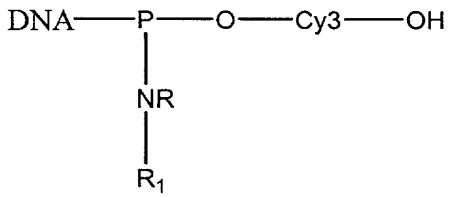
As an example illustrating the use of H-phosphonate chemistry in the synthesis of the CRE probes, the synthesis of five different charge-balanced CRE probes was performed (Fig. 11). All synthesized charge-balance probes were tested in an INVADER assay. It was found that the cleaved PCTs have different electrophoretic mobility under the conditions of reverse capillary electrophoresis.

The use of H-phosphonates in the modification of CRE probes is associated with the generation of a new center of chirality at the tetracoordinated phosphoramidate phosphorus atom (Fig. 12). The use of chiral (optically active) and more sterically bulky H-phosphonate monomers (e.g. dT, dA, dC, dG H-phosphonates) can lead to the formation of diastereoisomers, which will have different chromatographic and electrophoretic properties. When relatively small and achiral H-phosphonate monomer was used (*e.g.*, DMT–protected H-phosphonate of 1,6-hexanediol), the formation of the stereoisomers was not detectable under either reverse phase HPLC and capillary electrophoresis conditions. However, diastereoisomeric forms of the larger synthesized materials can be detected as separate peaks in the analytical RP HPLC profiles, and in the CE profiles of both the intact CRE probes and the positively charged products of enzymatic cleavage. The separations between diastereoisomers under those conditions can vary and can depend on the nature of the groups introduced in the modification step.

Introduction of multiple points (n) of modification using H-phosphonate reagents leads to the formation of 2^n diastereoisomers, which may or may not be separated under the conditions used for the probe purification, analysis or under the conditions of the CRE experiments. The separation of the diastereoisomers can be disadvantageous in situations where probes will be used in a multiplex assay. Formation of the diastereoisomeric forms of the charge balanced CRE probes was observed in all cases in which H-phosphonates of the 5'-DMT protected deoxynucleosides were used.

In one case, (dA H-phosphonate, amine used in the conversion of the intermediate H-phosphonate into the phosphoramidate: $H_2NCH_2CH_2NMe_2$) the separation of the diastereoisomers under reverse phase HPLC conditions (C-18 column) allowed separation of the isomers. Analysis of the isolated fractions by mass spectrometry revealed that the materials had identical molecular weight, corresponding to that of the desired product. Therefore, if a step of purifying the individual diastereoisomers is not intended, or when complete separation is not possible, the use of achiral H-phosphonates as a building block in the synthesis of the CRE probes for such system may be preferred to the use of chiral H-phosphonates. However, in cases when the separation of the diastereoisomers in pure form is possible (e.g., by reverse phase HPLC), the individual diastereoisomers can be used as separable tags in CRE assays, further expanding the diverse library of the H-phosphonate-generated CRE probes.

In some embodiments of the present invention, an H-phosphonate of Cy3 is used to directly introduce a charge-bearing unit into a charge tag. For example, use of an H-phosphonate of Cy3 can provide a charge tag containing the structure:



where any desired amine can be readily incorporated into the position NR. This allows, for example, the production of a palette of different charge tags that will provide different mobility in separation assays.

b. A New Class of Phosphoramidite Building Blocks:"Positively Charged Phosphoramidites" (PCP) and "Neutral Phosphoramidites" (NP).

Positively charged phosphoramidites (PCP) and neutral phosphoramidites (NP) represent a new class of phosphoramidite building blocks designed to introduce both positive charge and structure modulation into the synthesized charge-balanced CRE probe.

A standard coupling protocol using phosphoramidite reagents is associated with the introduction into the growing molecule, of one negative charge per coupling step, due to the formation of the phosphodiester linkage (Fig. 13). In the synthesis of charge-balanced CRE probes in which a specific ratio of negative and positive charges should be maintained, the introduction of additional negative charges can represent a disadvantage. To eliminate this disadvantage, new types of phosphoramidites were designed to either introduce a net positive charge(s) at each coupling step (positively charged phosphoramidites, PCPs), or to introduce no extra charge (neutral phosphoramidites, NPs) into the synthesized CRE probe. Fig. 14 shows general structures of the PCP and NP phosphoramidites in some embodiments of the present invention.

The positively charged group (Y^+) represents any organic group that can exist in a positively charged form, preferably primary, secondary or tertiary amines. Modification with the introduction of quarternary ammonium groups or other organic positively charged groups is also contemplated.

Both PCPs and NPs can be used in combination with other phosphoramidite building blocks (PBBs), which introduce one negative charge per coupling, but which can serve as structure modulating factors. Diversification of the structures of the PCPs and NPs can also serve as factors for the structure modulation of the synthesized CRE probe. This approach allows for the synthesis of a large variety of the charge-balanced CRE probes using a standard phosphoramidite coupling protocol for oligonucleotide synthesis.

For example, Fig. 15 illustrates possible combinations in the synthesis of the charge-balanced CRE probe when the synthesis is performed with the use of one dye phosphoramidite (DP), which introduces zero net charge (e.g., Cy3 phosphoramidite), PBB, which introduces one negative charge, one NP introducing zero net charge, and one PCP, which introduces one net positive charge. As shown in Fig. 15, due to the large

number of positional permutations possible in the design of the probe structure, a large variety of charge-balanced structures can be synthesized using only four reagents.

While Fig.15 illustrates the synthesis of the charge-balanced CRE probes in which the reporter molecule (Cy3) is attached directly to the oligonucleotide sequence, other structural permutations in which the reporter molecule can occupy other positions are also contemplated.

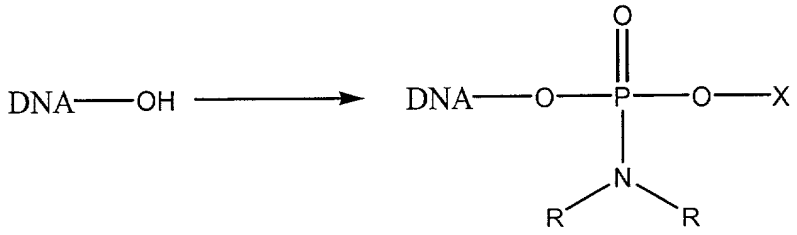
Therefore, this approach creates a unique opportunity to synthesize a large number of the charge-balanced CRE probes using only one reporter molecule. For example, Fig.13 presents an embodiment in which a dye that does not introduce any net charge (*e.g.*, Cy3 phosphoramidite) was used in probe synthesis. This does not preclude the use other dyes in the synthesis of a different set of charge-balanced CRE probes for use, *e.g.*, in multiplex detection systems using, for example, the INVADER Assay. It is also worth noting that, in contrast to the H-phosphonate approach, the use of the new type of phosphoramidites does not lead to the creation of new centers of chirality.

In an additional embodiment, the H-phosphonates and the phosphoramidites of the present invention are used in combination, *e.g.*, in the synthesis of the specifically modified charge-balanced CRE probes. Fig. 16 shows an example of the synthesized neutral phosphoramidite and positively charged phosphoramidite, and Fig. 17 shows the structures of a set of charge-balanced CRE probes that were synthesized utilizing PCPs and NPs.

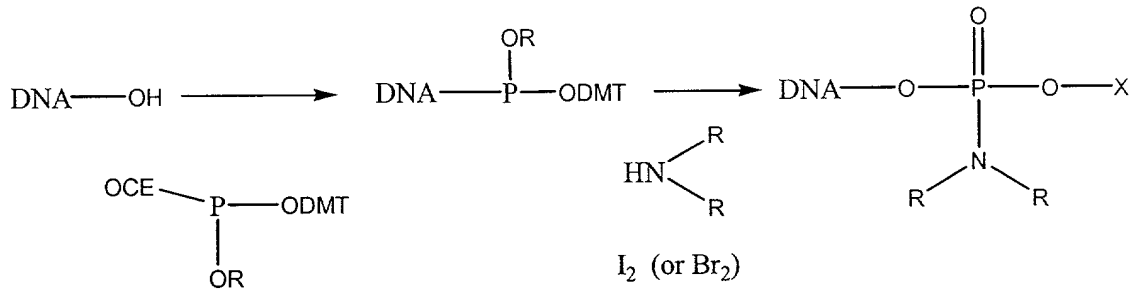
Commercially available phosphoramidite of the 18-atom linker (polyethylene glycol derivative; Glen Research; Cat.# 10-1918-90) was used as a building block phosphoramidite used for structure modulation, (indicated in Fig. 17 as "18AL").

Linkers of different lengths and of different chemical natures can be used as structure modulating reagents.

The present invention also provides new synthetic methods using phosphoramites to generate charge tags containing a unit with a charge group and a phosphate group. For example, as described above, H-phosphonate chemistry can be used to add a charged unit onto a nucleic acid structure:



(where X is one or more additional components of the charge tag and the R groups are any other desired chemical groups). The same structure may be generated using phosphoramidite addition by first adding the phosphoramite, then using a Michaelis-Arbuzov reaction in the presence of, for example, an amine:



The above methods of generating charge tags allow an extremely wide variety of charge tags to be made. This variety of options allows for multiplex detection methods. For example, in the context of the INVADER assay, a charge tag attached to a probe oligonucleotide could have three components:

3'-[probe]-5'-[Y_1-Y_2-Y_3]

where Y_1 is one of any number of dyes, Y_2 is one of any number of groups containing a positive charges, and Y_3 is one of four nucleotides (e.g., not complementary to the target

nucleic acid). If four different dyes and four different charged groups are used, this would introduce 4x4x4, or 64 distinct charge tags that could be individually resolvable using the methods described herein (e.g., microfluidics). By adding additional components or additional choices at each component, hundred to thousands or more distinct charge tags can be made and used in multiplex analyses.

EXAMPLES

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the disclosure which follows, the following abbreviations apply: Afu (Archaeoglobus fulgidus); Mth (Methanobacterium thermoautotrophicum); Mja (Methanococcus jannaschii); Pfu (Pyrococcus furiosus); Pwo (Pyrococcus woesei); Taq (Thermus aquaticus); Taq DNAP, DNAPTaq, and Taq Pol I (T. aquaticus DNA polymerase I); DNAPStf (the Stoffel fragment of DNAPTaq); DNAPEc1 (E. coli DNA polymerase I); Tth (Thermus thermophilus); Ex. (Example); Fig. (Figure);°C (degrees Centigrade); g (gravitational field); hr (hour); min (minute); olio (oligonucleotide); rxn (reaction); vol (volume); w/v (weight to volume); v/v (volume to volume); BSA (bovine serum albumin); CTAB (cetyltrimethylammonium bromide); HPLC (high pressure liquid chromatography); DNA (deoxyribonucleic acid); p (plasmid); µl (microliters); ml (milliliters); µg (micrograms); mg (milligrams); M (molar); mM (milliMolar); µM (microMolar); pmoles (picomoles); amoles (attomoles); zmoles (zeptomoles); nm (nanometers); kdal (kilodaltons); OD (optical density); EDTA (ethylene diamine tetra-acetic acid); FITC (fluorescein isothiocyanate); SDS (sodium dodecyl sulfate); $NaPO_4$ (sodium phosphate); NP-40 (Nonidet P-40); Tris (tris(hydroxymethyl)-aminomethane); PMSF (phenylmethylsulfonylfluoride); TBE (Tris-Borate-EDTA, i.e., Tris buffer titrated with boric acid rather than HCl and containing EDTA); PBS (phosphate buffered saline); PBS (phosphate buffered saline containing 1 mM PMSF); PAGE (polyacrylamide gel electrophoresis); Tween (polyoxyethylene-sorbitan); ATCC (American Type Culture Collection, Rockville, MD); Coriell (Coriell Cell Repositories, Camden, NJ); DSMZ (Deutsche Sammlung von Mikroorganismen und Zellculturen, Braunschweig, Germany); Sigma (Sigma Chemical Company, St. Louis,

MO); MJ Research (MJ Research, Watertown,MA); Novagen (Novagen, Inc., Madison, WI); Perkin Elmer (Perkin Elmer Instruments, Norwalk, CT); Promega (Promega Corp. Madison, WI); Clonetech (Clonetech, Palo Alto, CA); Pharmacia (Pharmacia, Piscataway, NJ); Hitachi (Hitachi Instruments Inc. San Jose, CA), Qiagen (Qiagen, Inc. Valencia, CA); Bio101 (Bio 101 Inc. Vista, CA); Aldrich (Aldrich Chemical Company Inc. Milwaukee, WI); VWR (VWR Scientific Products, West Chester, PA); Glen Research (Glen Research Corporation, Sterling VA); PE Biosystems (PE/ Applied Biosystems, Foster City, CA); Wheaton (Wheaton Science Products, Millville, NJ); EM Science (EM Science, Gibbstown NJ); Gelman (Gelman Science, Ann Arbor, MI); Becton Dickensen (Becton Dickensen Labware, Bedford, MA); Büchi (Büchi Analytical, Switzerland); Chemglass (Chemglass Inc. Vineland, NJ); Dot Scientific (Dot Scientific Inc. Burton, MI); Eppendorf Scientific (Eppendorf Scientific Inc. Westbury, NY); Applied Biosystems (Applied Biosystems, Foster City, CA); Invitrogen (Invitrogen Corporation, Carlsbad, CA); Ambion (Ambion Inc. Austin, TX); Gibco BRL (Life Technologies, Gaithersburg, MD); USB (US Biochemical, Cleveland, OH); Calbiochem (Calbiochem, San Diego, CA).

EXAMPLE 1

Detection Of DNA By Charge Reversal

The detection of specific targets is achieved in the INVADER-directed cleavage assay by the cleavage of a probe oligonucleotide. The cleaved probe may be separated from the uncleaved probe using the charge reversal technique described below. This novel separation technique is related to the observation that positively charged adducts can affect the electrophoretic behavior of small oligonucleotides because the charge of the adduct is significant relative to charge of the whole complex. Observations of aberrant mobility due to charged adducts have been reported in the literature, but in all cases found, the applications pursued by other scientists have involved making oligonucleotides larger by enzymatic extension. As the negatively charged nucleotides are added on, the positive influence of the adduct is reduced to insignificance. As a result, the effects of positively charged adducts have been dismissed and have received little notice in the existing literature.

Through the use of multiple positively charged adducts, synthetic molecules can be constructed with sufficient modification that the normally negatively charged strand is made nearly neutral. When so constructed, the presence or absence of a single phosphate group can mean the difference between a net negative or a net positive charge. This observation has particular utility when one objective is to discriminate between enzymatically generated fragments of DNA, which generally lack a 3' phosphate, and the products of thermal degradation, which generally retain a 3' phosphate (and thus two additional negative charges).

a) Characterization Of The Products Of Thermal Breakage Of DNA Oligonucleotides

Thermal degradation of DNA probes results in high background that can obscure signals generated by specific enzymatic cleavage, decreasing the signal-to-noise ratio. To better understand the nature of DNA thermal degradation products, the 5' tetrachloro-fluorescein (TET)-labeled oligonucleotides 78 (SEQ ID NO:3) and 79 (SEQ ID NO:4) (100 pmole each) were incubated in 50 µl 10 mM $NaCO_3$ (pH 10.6), 50 mM NaCl at 90°C for 4 hours. To prevent evaporation of the samples, the reaction mixture was overlaid with 50 µl of CHILLOUT liquid wax (MJ Research). The reactions were then divided in two equal aliquots (A and B). Aliquot A was mixed with 25 µl of methyl violet loading buffer and Aliquot B was dephosphorylated by addition of 2.5 µl of 100 mM $MgCl_2$ and 1 µl of 1 unit/µl Calf Intestinal Alkaline Phosphatase (CIAP) (Promega), with incubation at 37°C for 30 min. after which 25 µl of methyl violet loading buffer was added. One microliter of each sample was resolved by electrophoresis through a 12% polyacrylamide denaturing gel and imaged as described in Example 21; a 585 nm filter was used with the FMBIO Image Analyzer. The resulting imager scan is shown in Fig. 2.

In Fig. 2, lanes 1-3 contain the TET-labeled oligonucleotide 78 and lanes 4-6 contain the TET-labeled oligonucleotide 79. Lanes 1 and 4 contain products of reactions that were not heat treated. Lanes 2 and 5 contain products from reactions that were heat treated and lanes 3 and 6 contain products from reactions that were heat treated, then subjected to phosphatase treatment.

As shown in Fig. 2, heat treatment causes significant breakdown of the 5'-TET-labeled DNA, generating a ladder of degradation products (Fig. 2, lanes 2, 3, 5 and 6). Band intensities correlate with purine and pyrimidine base positioning in the oligonucleotide sequences, indicating that backbone hydrolysis may occur through formation of abasic intermediate products that have faster rates for purines than for pyrimidines (Lindahl and Karlström, Biochem., 12:5151 [1973]).

Dephosphorylation decreases the mobility of all products generated by the thermal degradation process, with the most pronounced effect observed for the shorter products (Fig. 2, lanes 3 and 6). This demonstrates that thermally degraded products possess a 3' end terminal phosphoryl group that can be removed by dephosphorylation with CIAP. Removal of the phosphoryl group decreases the overall negative charge by 2. Therefore, shorter products that have a small number of negative charges are influenced to a greater degree upon the removal of two charges. This leads to a larger mobility shift in the shorter products than that observed for the larger species.

The products generated by the CLEAVASE enzyme do not contain this additional 3' phosphate. Therefore, if an assay is designed such that the desired reaction products contain one or two positive charges, similar thermal breakdown products would be neutral or negative. This allows for easy separation of product from background via the reverse charge methods described below.

b) Dephosphorylation Of Short Amino-Modified Oligonucleotides Can Reverse The Net Charge Of The Labeled Product

To demonstrate how oligonucleotides can be transformed from net negative to net positively charged compounds, the four short amino-modified oligonucleotides labeled **70**, **74**, **75** and **76** and shown in Figs. 3-4 were synthesized. All four modified oligonucleotides possess Cy3 dyes positioned at the 5'-end, which individually are positively charged under reaction and isolation conditions described in this Example. Compounds **70** and **74** contain two amino modified thymidines that, under reaction conditions, display positively charged R-NH_3^+ groups attached at the C5 position through a C_{10} or C_6 linker, respectively. Because compounds **70** and **74** are 3'-end phosphorylated, they consist of four negative charges and three positive charges.

Compound **75** differs from **74** in that the internal C_6 amino modified thymidine phosphate in **74** is replaced by a thymidine methyl phosphonate. The phosphonate backbone is uncharged and so there are a total of three negative charges on compound **75**. This gives compound **75** a net negative one charge. Compound **76** differs from **70** in that the internal amino modified thymidine is replaced by an internal cytosine phosphonate. The pK_a of the N3 nitrogen of cytosine can be from 4 to 7. Thus, the net charges of this compound, can be from -1 to 0 depending on the pH of the solution. For the simplicity of analysis, each group is assigned a whole number of charges, although it is realized that, depending on the pK_a of each chemical group and ambient pH, a real charge may differ from the whole number assigned. It is assumed that this difference is not significant over the range of pHs used in the enzymatic reactions studied here.

Dephosphorylation of these compounds, or the removal of the 3' end terminal phosphoryl group, results in elimination of two negative charges and generates products that have a net positive charge of one. In this experiment, the method of isoelectric focusing (IEF) was used to demonstrate a change from one negative to one positive net charge for the described substrates during dephosphorylation.

Substrates **70**, **74**, **75** and **76** were synthesized by standard phosphoramidite chemistries and deprotected for 24 hours at 22°C in 14 M aqueous ammonium hydroxide solution, after which the solvent was removed in vacuo. The dried powders were resuspended in 200 µl of H_2O and filtered through 0.2 µm filters. The concentration of the stock solutions was estimated by UV-absorbance at 261 nm of samples diluted 200-fold in H_2O using a spectrophotometer (Spectronic Genesys 2, Milton Roy, Rochester, NY).

Dephosphorylation of compounds **70** and **74**, **75** and **76** was accomplished by treating 10 µl of the crude stock solutions (ranging in concentration from approximately 0.5 to 2 mM) with 2 units of CIAP in 100 µl of CIAP buffer (Promega) at 37°C for 1 hour. The reactions were then heated to 75°C for 15 min. in order to inactivate the CIAP. For clarity, dephosphorylated compounds are designated 'dp'. For example, after dephosphorylation, substrate **70** becomes **70dp**.

To prepare samples for IEF experiments, the concentration of the stock solutions of substrate and dephosphorylated product were adjusted to a uniform absorbance of 8.5

x 10^{-3} at 532 nm by dilution with water. Two microliters of each sample were analyzed by IEF using a PhastSystem electrophoresis unit (Pharmacia) and PhastGel IEF 3-9 media (Pharmacia) according to the manufacturer's protocol. Separation was performed at 15°C with the following program: pre-run; 2,000 V, 2.5 mA, 3.5 W, 75 Vh; load; 200 V, 2.5 mA, 3.5 W, 15 Vh; run; 2,000 V; 2.5 mA; 3.5 W, 130 Vh. After separation, samples were visualized by using the FMBIO Image Analyzer (Hitachi) fitted with a 585 nm filter. The resulting imager scan is shown in Fig. 18.

Fig. 18 shows results of IEF separation of substrates **70**, **74**, **75** and **76** and their dephosphorylated products. The arrow labeled "Sample Loading Position" indicates a loading line, the '+' sign shows the position of the positive electrode and the '-' sign indicates the position of the negative electrode.

The results shown in Fig. 18 demonstrate that substrates **70**, **74**, **75** and **76** migrated toward the positive electrode, while the dephosphorylated products **70dp**, **74dp**, **75dp** and **76dp** migrated toward negative electrode. The observed difference in mobility direction was in accord with predicted net charge of the substrates (minus one) and the products (plus one). Small perturbations in the mobilities of the phosphorylated compounds indicate that the overall pIs vary. This was also true for the dephosphorylated compounds. The presence of the cytosine in **76dp**, for instance, moved this compound further toward the negative electrode, which was indicative of a higher overall pI relative to the other dephosphorylated compounds. It is important to note that additional positive charges can be obtained by using a combination of natural amino modified bases (**70dp** and **74dp**) along with uncharged methylphosphonate bridges (products **75dp** and **76dp**).

The results shown above demonstrate that the removal of a single phosphate group can flip the net charge of an oligonucleotide to cause reversal in an electric field, allowing easy separation of products, and that the precise base composition of the oligonucleotides affect absolute mobility but not the charge-flipping effect.

EXAMPLE 2

Detection Of Specific Cleavage Products In The INVADER-Directed Cleavage Reaction By Charge Reversal

In this Example the ability to isolate products generated in the INVADER-directed cleavage assay from all other nucleic acids present in the reaction cocktail using charge reversal is demonstrated.

Enzymes for Cleavage Assays

The CLEAVASE A/G enzyme was prepared as described in U.S. Patent 6,090,606, and PCT application WO 98/23774 (herein incorporated by reference in their entireties); *Afu* FEN 1 and *Pfu* FEN1 were isolated as described in WO 98/23774. Two other enzymes used in these studies, CLEAVASE TthAKK enzyme and *Ave* FEN1 nuclease, were produced as described in the following sections.

Cloning and Expression of Cleavase TthAKK

Initial TthPol Isolation

Genomic DNA was prepared from 1 vial of dried *Thermus thermophilus* strain HB-8 from ATCC (ATCC #27634). The DNA polymerase gene was amplified by PCR using the following primers: 5'-CACGAATTCCGAGGCGATGCTTCCGCTC-3' (SEQ ID NO:5) and 5'-TCGACGTCGACTAACCCTTGGCGGAAAGCC-3' (SEQ ID NO:6). The resulting PCR product was digested with *Eco*RI and *Sal*I restriction endonucleases and inserted into *Eco*RI/*Sal* I digested plasmid vector pTrc99G . The pTrc99G vector was created by modification of the pTrc99A vector (Pharmacia) to remove the G at position 270 of the pTrc99A map. To this end, pTrc99A plasmid DNA was cut with *Nco*I and the recessive 3' ends were filled-in using the Klenow fragment of *E.coli* polymerase I in the presence of all four dNTPs at 37°C for 15 min. After inactivation of the Klenow fragment by incubation at 65°C for 10 min, the plasmid DNA was cut with *Eco*RI and the ends were again filled-in using the Klenow fragment in the presence of all four dNTPs at 37°C for 15 min. The Klenow fragment was then inactivated by incubation at 65°C for 10 min. The plasmid DNA was ethanol precipitated, recircularized by ligation, and used to transform *E.coli* JM109 cells (Promega). The pTrc99G plasmid DNA was isolated from single colonies, and deletion of the G at position 270 (by reference to the pTrc99A map) was confirmed by DNA sequencing.

Insertion of the Tth DNA into this vector as described above created the plasmid pTrcTth-1. This *Tth* polymerase construct is missing a single nucleotide that was inadvertently omitted from the 5' oligonucleotide, resulting in the polymerase gene being out of frame. This mistake was corrected by site specific mutagenesis of pTrcTth-1 using the TRANSFORMER Site Directed Mutagenesis Kit (Clontech) according to the manufacturer's instructions, and the following oligonucleotide: 5'-GCATCGCCTCGGAATTCATGGTC-3' (SEQ ID NO:7), to create the plasmid pTrcTth-2. The protein and the nucleic acid sequence encoding the protein are referred to as TthPol, and are listed as SEQ ID NOS:8 and 9 respectively.

Modified TthPol Gene: Tth DN

The *Tth* DN construct was created by mutating the TthPol-2 described above. The sequence encoding an aspartic acid at position 787 was changed by site-specific mutagenesis as described above to a sequence encoding asparagine. Mutagenesis of pTrcTth-2 with the following oligonucleotide: 5'-CAGGAGGAGCTCGTTGTGGACCTGGA-3' (SEQ ID NO:10) was performed to create the plasmid pTrcTthDN. The mutant protein, termed Tth DN, and protein coding nucleic acid sequence are SEQ ID NOS:11 and 12, respectively.

Tth DN HT

A six-amino acid histidine tag (his-tags) was added onto the carboxy terminus of Tth DN. The site-directed mutagenesis was performed using the TRANSFORMER Site Directed Mutagenesis Kit (Clontech) according to the manufacturer's instructions. The mutagenic oligonucleotides used on the plasmid pTth DN was sequence 5'-TGCCTGCAGGTCGACGCTAGCTAGTGGTGGTGGTGGTGGTGACCCTTGGCG GAAAGCC-3' (SEQ ID NO:13), sequence 136-037-05. The selection primer Trans Oligo AlwNI/SpeI (Clontech, catalog # 6488-1) was used for both mutagenesis reactions. The resulting mutant gene was termed Tth DN HT (SEQ ID NO:14, nucleic acid sequence; SEQ ID NO:15, amino acid sequence).

Purification of Tth DN HT

The Tth DN HT protein was expressed in *E. coli* strain JM109 as described above. After ammonium sulfate precipitation and centrifugation, the protein pellet was suspended in 0.5 ml of Q buffer (50 mM Tris-HCl, pH 8.0, 0.1 mM EDTAm 0.1% Tween 20). The protein was further purified by affinity chromatography using His-Bind Resin and Buffer Kit (Novagen) according to the manufacturer's instructions. 1 ml of His-Bind resin was transferred into a column, washed with 3 column volumes of sterile water, charged with 5 volumes of 1X Charge Buffer, and equilibrated with 3 volumes of 1X Binding Buffer. Four ml of 1X Binding Buffer was added to the protein sample and the sample solution was loaded onto the column. After washing with 3 ml of 1X Binding Buffer and 3 ml of 1X Wash Buffer, the bound His-Tag protein was eluted with 1 ml of 1X Elute Buffer. The pure enzyme was then dialyzed in 50% glycerol, 20 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.5% Tween 20, 0.5% Nonidet P40, and 100 μg.ml BSA. Enzyme concentrations were determined by measuring absorption at 279 mn.

Generation of Tth DN RX HT

Mutagenesis was performed to introduce 3 additional, unique restriction sites into the polymerase domain of the Tth DN HT enzyme. Site specific mutagenesis was performed using the Transformer Site-Directed Mutagenesis Kit from (Clontech) according to manufacturer's instructions. One of two different selection primers, Trans Oligo AlwNI/SpeI or Switch Oligo SpeI/AlwNI (Clontech catalog #6488-1 or catalog #6373-1) was used for all mutagenesis reactions described. The selection oligo used in a given reaction is dependent on the selection restriction site present in the vector. All mutagenic primers were synthesized by standard synthetic chemistry. Resultant colonies were expressed in *E.coli* strain JM109.

The Not I site (amino acid position 328) was created using the mutagenic primer 5'-GCCTGCAGGGGCGGCCGCGTGCACCGGGGCA (SEQ ID NO:16) corresponding to the sense strands of the Tth DN HT gene. The BstI (amino acid position 382) and NdeI (amino acid position 443) sites were introduced using sense strand mutagenic primers 5'-CTCCTGGACCCTTCGAACACCACCCC (SEQ ID NO:17) and 5'-GTCCTGGCCCATATGGAGGCCAC (SEQ ID NO:18), respectively. The mutant

plasmid was over-expressed and purified using Qiagen QiaPrep Spin Mini Prep Kit (cat. # 27106). The vector was tested for the presence of the restriction sites by DNA sequencing and restriction mapping. The construct is termed Tth DN RX HT (DNA sequence SEQ ID NO:19; amino acid sequence SEQ ID NO:20)

Addition of point mutations

Plasmid DNA was purified from 200 ml of JM109 overnight culture using QIAGEN Plasmid Maxi Kit (QIAGEN) according to the manufacturer's protocol to obtain enough starting material for all mutagenesis reactions. All site-specific mutations were introduced using the Transformer Site Directed mutagenesis Kit (Clontech) according to the manufacturer's protocol. One of two different selection primers, Trans Oligo AlwNI/SpeI or Switch Oligo SpeI/AlwNI (Clontech, Palo Alto CA catalog #6488-1 or catalog #6373-1) was used for all mutagenesis reactions described. The selection oligo used in a given reaction is dependent on the restriction site present in the vector. All mutagenic primers were synthesized by standard synthetic chemistry. Resultant colonies for both types of reactions were *E.coli* strain JM109. Expression and purification of the mutant protein was done as detailed above.

Construction of Tth DN RX HT H786A

Site specific mutagenesis was performed on pTrc99G Tth DN RX HT DNA using the mutagenic primer 583-001-04: 5'-CAG GAG GAG CTC GTT GGC GAC CTG GAG GAG-3' (SEQ ID NO:21) to generate the H786A mutant enzyme (DNA sequence SEQ ID NO:22; amino acid sequence SEQ ID NO:23).

Construction of Tth DN RX HT (H786A/G506K/Q509K)

Starting with the mutant Tth DN RX HT H786A, generated above, site specific mutagenesis was done using the mutagenic primer 604-022-02: 5'-GGA GCG CTT GCC TGT CTT CTT CGT CTT CTT CAA GGC GGG AGG CCT-3' (SEQ ID NO:24) to generate this variant termed "Cleavase TthAKK", (DNA sequence SEQ ID NO:25; amino acid sequence SEQ ID NO:26).

Large Scale preparation of recombinant proteins

The recombinant proteins were purified by the following technique which is derived from a *Taq* DNA polymerase preparation protocol (Engelke *et al.*, Anal. Biochem., 191:396 [1990]) as follows. *E. coli* cells (strain JM109) containing either pTrc99A TaqPol, pTrc99GTthPol were inoculated into 3 ml of LB containing 100 mg/ml ampicillin and grown for 16 hrs at 37°C. The entire overnight culture was inoculated into 200 ml or 350 ml of LB containing 100 mg/ml ampicillin and grown at 37°C with vigorous shaking to an A_{600} of 0.8. IPTG (1 M stock solution) was added to a final concentration of 1 mM and growth was continued for 16 hrs at 37°C.

The induced cells were pelleted and the cell pellet was weighed. An equal volume of 2X DG buffer (100 mM Tris-HCl, pH 7.6, 0.1 mM EDTA) was added and the pellet was suspended by agitation. Fifty mg/ml lysozyme (Sigma) were added to 1 mg/ml final concentration and the cells incubated at room temperature for 15 min. Deoxycholic acid (10% solution) was added dropwise to a final concentration of 0.2 % while vortexing. One volume of H_2O and 1 volume of 2X DG buffer were added, and the resulting mixture was sonicated for 2 minutes on ice to reduce the viscosity of the mixture. After sonication, 3 M $(NH_4)_2SO_4$ was added to a final concentration of 0.2 M, and the lysate was centrifuged at 14000 x *g* for 20 min at 4°C. The supernatant was removed and incubated at 70°C for 60 min at which time 10% polyethylimine (PEI) was added to 0.25%. After incubation on ice for 30 min., the mixture was centrifuged at 14,000 x *g* for 20 min at 4°C. At this point, the supernatant was removed and the protein precipitated by the addition of $(NH_4)_2SO_4$ as follows.

Two volumes of 3 M $(NH_4)_2SO_4$ were added to precipitate the protein. The mixture was incubated overnight at room temperature for 16 hrs centrifuged at 14,000 x *g* for 20 min at 4°C. The protein pellet was suspended in 0.5 ml of Q buffer (50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 0.1% Tween 20). The suspended protein preparations were quantitated by determination of the A_{279} dialyzed and stored in 50% glycerol, 20 mM Tris HCl, pH8.0, 50 mM KCl, 0.5% Tween 20, 0.5% Nonidet P-40, with 100 µg/ml BSA.

Cloning and Expression of AveFEN1 Nuclease

A common method for cloning new members of a gene family is to run PCR reactions using degenerate oligonucleotides complementary to conserved amino acid sequences in that family, and then to clone and sequence the gene-specific PCR fragments. This sequence information can then be used to design sense and anti-sense gene-specific primers which can be used in PCR walking reactions (Nucleic Acids Res. 1995a. 23(6)1087-1088) to obtain the remainder of the gene sequence. The sequences obtained from the sense and anti-sense PCR walks can then be combined to generate the DNA sequence for the entire open reading frame (ORF) of the gene of interest. Once the entire ORF is known, primers specific to both the 5' and the 3' end of the gene can be designed, and PCR reactions can be performed on genomic DNA to amplify the gene in its entirety. This organism-specific, amplified fragment can then be cloned into an expression vector, and via methods know in the art, and detailed below, the protein of interest can be expressed and purified.

A. Degenerate PCR and PCR walking to obtain the sequence of the Ave FEN1 gene

The protein sequences of the FEN1 genes from *Pyrococcus furiosus* (SEQ ID NO:27) *Methanococcus jannaschii* (SEQ ID NO:28), *Methanobacterium thermoautotrophicum* (SEQ ID NO:29), and *Archaeoglobus fulgidus* (SEQ ID NO:30) were aligned and blocks of conserved amino acids were identified. The conserved sequence blocks VFDG (valine, phenylalanine, aspartic acid, glycine), EGEAQ (glutamic acid, glycine, glutamic acid, alanine, glutamine), SQDYD (serine, glutamine, aspartic acid, tyrosine, aspartic acid), and GTDYN/GTDFN (glycine, threonine, aspartic acid, tyrosine or phenylalanine, asparagine) were chosen as sequences that would likely be present in all Archaeal FEN1 genes. Degenerate oligonucleotides were designed for each of these conserved sequence blocks. In addition to the FEN1 gene specific portion of the oligonucleotides a 15-nucleotide tail was added to the 5' end of the oligonucleotides to enable nested PCR. A different tail sequence was used depending on whether the degenerate oligonucleotide targets the sense or antisense strand of the FEN1 gene.

Forward and/or reverse versions of the oligonucleotides were made and target the sense and antisense strands of the FEN1 gene respectively. The oligonucleotides are VFDG-Fwd (SEQ ID NO:31), EGEAQ-Fwd (SEQ ID NO:32) QDYD-Fwd (SEQ ID NO:33), EGEAQ-Rev (SEQ ID NO:34), SQDYD-Rev1 (SEQ ID NO:35), SQDYD-Rev2 (SEQ ID NO:36), and GTDYN-Rev (SEQ ID NO:37). Two oligonucleotides were made for the SQDYD-Rev sequence because serine is encoded by 6 different codons. For use in PCR, the SQDYD-Rev1 and SQDYD-Rev2 oligonucleotides were mixed in a ratio of 1:2. For the QDYD-Fwd oligonucleotide, the requirement for mixing was avoided by targeting only the last four amino acids of the conserved SQDYD sequence. The GTDYN-Rev oligonucleotide also recognizes the sequence GTDFN since the codons for tyrosine and phenylalanine share 2 of 3 nucleotides.

First, genomic DNA was prepared from 1 vial of the live bacterial strain as described below. All bacterial strains were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Acidianus ambivalens– DSM # 3772). When the cells were lyophilized, they were resuspended in 200 µl of TNE (10 mM TrisHCL, pH 8.0, 1 mM EDTA, 100 mM NaCl). When the cells were in liquid suspension, they were spun down at 20,000 x G for 2 minutes and the cell pellets were resuspended in 200 µl of TNE. 20 µl of 20% SDS (sodium dodecylsulfate) and 2 µl of 1 mg/ml proteinase K were added and the suspension was incubated at 65°C for 30 minutes. The lysed cell suspension was extracted in sequential order with buffered phenol, 1:1 phenol: chloroform, and chloroform. The nucleic acid was precipitated by the addition of on equal volume of cold 100% ethanol. The nucleic acid was pelleted by spinning at 20,000 x G for 5 minutes. The nucleic acid pellet was washed with 70% ethanol, air dryed and resuspended in 50 µl of TE (10 mM TrisHCL, pH 8.0, 1 mM EDTA). The final DNA pellet was re-suspended in 50 µl of TE (10 mM Tris HCl, pH 8.0, 1 mM EDTA).

Both reactions of the nested PCR were done using the Advantage cDNA PCR kit (Clontech) according to manufacturer's instructions using a final concentration of 1 µM for all oligonucleotides. The first reaction is done in a 20 µl volume with one of the 6 possible combinations of forward and reverse degenerate oligonucleotides, and includes either 1 µl of the genomic DNA preparation described above. The cycling conditions

were 20 cycles of 95°C for 15 seconds, 50°C or 55°C for 15 seconds, and 68°C for 30 seconds. The second reactions utilize primers that have the same sequence as the 5' tail sequence of the degenerate oligonucleotides described above. The two primers are 203-01-01 (SEQ ID NO:38) and 203-01-02 (SEQ ID NO:39). The second reaction is carried out exactly as described for the first reaction, except 30 cycles are done instead of 20 and the reaction volume is 25 µl. Following the second PCR, 5 µl of the reaction were loaded on a 2% or 4% agarose gel and the DNA was visualized by ethidium bromide staining. The expected product sizes based on the previously identified FEN1 sequences for all primer pairs are as follows: VFDG-Fwd and EGEAQ-Rev; 275 base pairs, VFDG-Fwd and SQDYD-Rev; 325 base pairs, VFDG Fwd and GTDYN-Rev; 510 base pairs, EGEAQ-Fwd and SQDYD-Rev; 100 base pairs, EGEAQ-Fwd and GTDYN-Rev; 290 base pairs, QDYD-Fwd and GTDYN-Rev; 230 base pairs. The primer pair, VFDG-Fwd and EGEAQ-Rev was able to generate a correctly sized DNA product for all samples attempted. The primer pair, VFDG-Fwd and GTDYN-Rev was able to generate a correctly sized DNA product for most of the DNA samples attempted.

When a DNA product of the expected size was made by the degenerate PCR, that DNA fragment was isolated and cloned into pGEM-T Easy (Promega) using the pGEM-T Easy ligation kit according to the manufacturer's instructions. The DNA sequence was determined and the sequence was used to generate sense and antisense genome walking oligonucleotides for cloning the remainder of the FEN1 gene. The oligonucleotides were designed according to the parameters of the GenomeWalker kit (Clontech) which was used prepare the various genomic DNA samples for the genome walking PCR reactions.

The genomic DNA was randomly amplified using a random 12-mer oligonucleotide. One hundred- µl PCR reactions were set up with the Advantage cDNA PCR kit (Clontech) and contained 10 µl of genomic DNA and 15 µM random 12-mer oligonucleotide. 50 cycles were carried out with the following parameters: 95°C for 30 seconds, 50°C for 30 seconds, 68°C for 5 minutes. After the PCR reactions were complete, amplified DNA was purified with the High Pure PCR Product Purification kit (Boehringer Mannheim). The purified DNA was eluted into a total of 200 µl of 10 mM TrisHCL, pH 8.5.

The genome walking protocol consists of 3 steps. First, a genomic DNA sample is cut with 5 different blunt-end restriction enzymes in 5 separate reactions. Second, the cut DNA is ligated to an adapter which serves as a tag sequence and also is designed to prevent background amplification. Third, the ligated DNA is amplified with a gene-specific primer and a primer with the same sequence as a portion of the adapter sequence.

50µl restriction digests contained 30 µl of randomly amplified genomic DNA and the Dra I restriction enzyme. After 4 hours at 37°C, the cut DNA was purified with either GENECLEANII (Bio 101) or QIAEX II (Qiagen) according to manufacturer's instructions. DNA was eluted into 10 µl of 10 mM TrisHCl, pH 8.5 in either case. 5.6µl of this cut DNA was used in 10 µl ligation reactions containing 6µM GenomeWalker adapter. Reactions were carried out at room temperature overnight followed by heating at 70°C for 10 minutes to inactivate the T4 DNA ligase. The ligation reactions were then diluted with 70 µl of TE (10 mM TrisHCl, pH 8.0, 1 mM EDTA).

One µl of the diluted ligation mix was used in 25 µl PCR reactions with 0.2 µM gene-specific primer and 0.2 µM primer AP-1 (Clontech) which has the same sequence as the 5' portion of the GenomeWalker adapter. Ten reactions were done for each DNA sample. Five antisense walk PCR reactions (for the 5 different restriction enzymes used to cut the genomic sample) were done using the sense gene-specific primer and five sense walk PCR reactions were done using the antisense gene-specific primer for each DNA sample. The cycling parameters were as recommended by the Universal Genome Walking kit (Clontech) and were as follows: 7 cycles of 94°C for 25 seconds and 72°C for 3 minutes, 32 cycles of 94°C for 25 seconds and 67°C for 3 minutes, followed by 67°C for 7 minutes. .

The *Archaeoglobus veneficus* (Ave) genome walks were done as follows. The primary antisense primer was Ave 34AS (SEQ ID NO:40) and the primary sense primer was Ave 65S (SEQ ID NO:41). Nested PCR reactions were done using the nested primer AP-2 and either the nested antisense primer Ave 32AS (SEQ ID NO:42) or the nested sense primer Ave 67S (SEQ ID NO:43). 25-µl nested reactions were done as descibed above for the primary PCR walk reactions. The primary reactions were diluted 1:50 in H_2O and 0.5 µl of those dilutions were added to the nested PCR reactions. The cycling parameters for the nested PCR reactions were as recommended by the Universal Genome

Walking kit (Clontech) and are as follows: 5 cycles of 94°C for 25 seconds and 72°C for 3 minutes, 20 cycles of 94°C for 25 seconds and 67°C for 3 minutes, followed by 7 minutes at 67°C. The nested antisense PCR reaction on Stu I cut Ave genomic sample generated a 1 kilobase DNA product which was cloned into pGEM-T Easy (Promega) following manufacturer's instructions and sequenced. The nested sense PCR reaction on Eco RV cut Ave genomic sample generated a 1.1 kilobase product which was cloned into pGEM-T Easy (Promega) following manufacturer's instructions and sequenced.

Cloning of Ave FEN-1 nuclease I into an expression vector

PCR reactions were performed using the primers designed above and genomic DNA from the organism of interest. The PCR products were gel purified and then cut with restriction endonucleases corresponding to the sites incorporated in the PCR primers. The cut PCR products were then purified away from the smaller digest fragments and these cut products were cloned into an expression vector. In some cases, this was the final step of the cloning process, prior to transformation and protein expression/purification. In some cases a fifth step was needed. In some cases, a mutagenesis step had to be performed to remove any nucleotides that were incorporated into the ORF as a result of primer sequences required for cloning.

Finally, a bacterial host (e.g., E. coli JM109) was transformed with the expression vector containing the cloned FEN-1, and protein expression and purification were done as detailed below.

The cloning of a FEN-1 from *Archaeaglobus veneficus* (Ave) was performed as described above using the DSM # 11195 genomic DNA and PCR primers Ave 5' – 3' TAACGAATTCGGTGCAGACATAGGCGAACTAC (SEQ ID NO:44) and Ave 3' – 5' CGGTGTCGACTCAGGAAAACCACCTCTCAAGCG (SEQ ID NO:45). The mutagenic oligonucleotide used was Ave ΔR1 – 5' CACAGGAAACAGACCATGGGTGCAGACATAGGCGAAC (SEQ ID NO:46). The open reading frame (ORF) encoding the *Ave* FEN-1 endonuclease is provided in SEQ ID NO:47; the amino acid sequence encoded by this ORF is provided in SEQ ID NO:48.

Large Scale Preparation of Recombinant Ave FEN-1 Protein

Ave FEN-1 protein was purified by the following technique, which is derived from a *Taq* DNA polymerase preparation protocol (Engelke *et al.*, Anal. Biochem., 191:396 [1990]) as follows. *E. coli* cells (strain JM109) containing the construct described above were inoculated into 3 ml of LB (Luria Broth) containing 100 μg/ml ampicillin and grown for 16 hrs at 37°C. The entire overnight culture was inoculated into 200 ml or 350 ml of LB containing 100 μg/ml ampicillin and grown at 37°C with vigorous shaking to an A_{600} of 0.8. IPTG (1 M stock solution) was added to a final concentration of 1 mM and growth was continued for 16 hrs at 37°C.

The induced cells were pelleted and the cell pellet was weighed. An equal volume of 2X DG buffer (100 mM Tris-HCl, pH 7.6, 0.1 mM EDTA) was added and the pellet was resuspended by agitation. Fifty mg/ml lysozyme (Sigma, St. Louis, MO) was added to 1 mg/ml final concentration and the cells were incubated at room temperature for 15 min. Deoxycholic acid (10% solution) was added dropwise to a final concentration of 0.2 % while vortexing. One volume of H_2O and 1 volume of 2X DG buffer was added and the resulting mixture was sonicated for 2 minutes on ice to reduce the viscosity of the mixture. After sonication, 3 M $(NH_4)_2SO_4$ was added to a final concentration of 0.2 M and the lysate was centrifuged at 14000 x *g* for 20 min at 4°C. The supernatant was removed and incubated at 70°C for 60 min at which time 10% polyethylimine (PEI) was added to 0.25%. After incubation on ice for 30 min., the mixture was centrifuged at 14,000 x *g* for 20 min at 4°C. At this point, the supernatant was removed and the FEN-1 protein was precipitated by the addition of $(NH_4)_2SO_4$ as follows.

The FEN-1 protein was precipitated by the addition of solid $(NH_4)_2SO_4$ to a final concentration of 3 M (~75% saturated). The mixture was incubated on ice for 30 min and the protein was centrifuged at 14,000 x g for 20 min at 4°C. The protein pellet was resuspended in 0.5 ml of Q buffer (50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 0.1% Tween 20). The resuspended protein preparations were quantitated by determination of the A_{279} .

INVADER assay using charged-balanced probes

This experiment utilized the following Cy3-labeled oligonucleotide: 5'-Cy3-AminoT-AminoT-CTTTTCACCAGCGAGACGGG-3' (SEQ ID NO:1; termed "oligo 61"). Oligo 61 was designed to release upon cleavage a net positively charged, labeled product. To test whether or not a net positively charged 5'-end labeled product would be recognized by the CLEAVASE enzymes in the INVADER-directed cleavage assay format, probe oligo 61 (SEQ ID NO:1) and INVADER oligonucleotide 67 (SEQ ID NO:2) were chemically synthesized on a DNA synthesizer (ABI 391) using standard phosphoramidite chemistries and reagents obtained from Glen Research (Sterling, VA).

Each assay reaction comprised 100 fmoles of M13mp18 single stranded DNA, 10 pmoles each of the probe (SEQ ID NO:1) and INVADER (SEQ ID NO:2) oligonucleotides, and 20 units of CLEAVASE A/G in a 10 µl solution of 10 mM MOPS, pH 7.4 with 100 mM KCl. Samples were overlaid with mineral oil to prevent evaporation. The samples were brought to 50°C, 55°C, 60°C, or 65°C and cleavage was initiated by the addition of 1 µl of 40 mM $MnCl_2$. Reactions were allowed to proceed for 25 minutes and then were terminated by the addition of 10 µl of 95% formamide containing 20 mM EDTA and 0.02% methyl violet. The negative control experiment lacked the target M13mp18 and was run at 60°C. Five microliters of each reaction were loaded into separate wells of a 20% denaturing polyacrylamide gel (cross-linked 29:1) with 8 M urea in a buffer containing 45 mM Tris-Borate (pH 8.3) and 1.4 mM EDTA. An electric field of 20 watts was applied for 30 minutes, with the electrodes oriented as indicated in Fig. 19B (*i.e.*, in reverse orientation). The products of these reactions were visualized using the FMBIO fluorescence imager and the resulting imager scan is shown in Fig. 19B.

Fig. 19A provides a schematic illustration showing an alignment of the INVADER (SEQ ID NO:2) and probe (SEQ ID NO:1) along the target M13mp18 DNA; only 53 bases of the M13mp18 sequence is shown (SEQ ID NO:49). The sequence of the INVADER oligonucleotide is displayed under the M13mp18 target and an arrow is used above the M13mp18 sequence to indicate the position of the INVADER relative to the probe and target. As shown in Fig. 19A, the INVADER and probe oligonucleotides share a 2 base region of overlap.

In Fig. 19B, lanes 1-4 contain reactions performed at 50°C, 55°C, 60°C, and 65°C, respectively; lane 5 contained the control reaction (lacking target). In Fig. 19B, the products of cleavage are seen as dark bands in the upper half of the panel; the faint lower band seen appears in proportion to the amount of primary product produced and, while not limiting the invention to a particular mechanism, may represent cleavage one nucleotide into the duplex. The uncleaved probe does not enter the gel and is thus not visible. The control lane showed no detectable signal over background (lane 5). As expected in an invasive cleavage reaction, the rate of accumulation of specific cleavage product was temperature-dependent. Using these particular oligonucleotides and target, the fastest rate of accumulation of product was observed at 55°C (lane 2) and very little product observed at 65°C (lane 4).

When incubated for extended periods at high temperature, DNA probes can break non-specifically (*i.e.*, suffer thermal degradation) and the resulting fragments contribute an interfering background to the analysis. The products of such thermal breakdown are distributed from single-nucleotides up to the full length probe. In this experiment, the ability of charge based separation of cleavage products (*i.e.*, charge reversal) would allow the sensitive separation of the specific products of target-dependent cleavage from probe fragments generated by thermal degradation was examined.

To test the sensitivity limit of this detection method, the target M13mp18 DNA was serially diluted ten fold over than range of 1 fmole to 1 amole. The INVADER and probe oligonucleotides were those described above (*i.e.*, SEQ ID NOS:2 and 1, respectively). The invasive cleavage reactions were run as described above with the following modifications: the reactions were performed at 55°C, 250 mM or 100 mM KGlu was used in place of the 100 mM KCl and only 1 pmole of the INVADER oligonucleotide was added. The reactions were initiated as described above and allowed to progress for 12.5 hours. A negative control reaction that lacked added M13m18 target DNA was also run. The reactions were terminated by the addition of 10 µl of 95% formamide containing 20 mM EDTA and 0.02% methyl violet, and 5 µl of these mixtures were electrophoresed and visualized as described above. The resulting imager scan is shown in Fig. 20.

In Fig. 20, lane 1 contains the negative control; lanes 2-5 contain reactions performed using 100 mM KGlu; lanes 6-9 contain reactions performed using 250 mM KGlu. The reactions resolved in lanes 2 and 6 contained 1 fmole of target DNA; those in lanes 3 and 7 contained 100 amole of target; those in lanes 4 and 8 contained 10 amole of target and those in lanes 5 and 9 contained 1 amole of target. The results shown in Fig. 20 demonstrate that the detection limit using charge reversal to detect the production of specific cleavage products in an invasive cleavage reaction is at or below 1 attomole or approximately 6.02×10^5 target molecules. No detectable signal was observed in the control lane, which indicates that non-specific hydrolysis or other breakdown products do not migrate in the same direction as enzyme-specific cleavage products. The excitation and emission maxima for Cy3 are 554 and 568, respectively, while the FMBIO Imager Analyzer excites at 532 and detects at 585. Therefore, the limit of detection of specific cleavage products can be improved by the use of more closely matched excitation source and detection filters.

EXAMPLE 3

Examination Of The Effects Of A 5' Positive Charge On The Rate Of Invasive Cleavage Using The CLEAVASE A/G Or *Pfu* FEN-1 Nucleases

To investigate whether the positive charges on the 5' ends of probe oligonucleotides containing a positively charged adduct(s) have an effect on the ability of the CLEAVASE A/G or *Pfu* FEN-1 nucleases to cleave the 5' arm of the probe, the following experiment was performed.

Two probe oligonucleotides having the following sequences were utilized in INVADER reactions: Probe 34-180-1: (N-Cy3)$T_{NH2}T_{NH2}$CCAGAGCCTAATTTGCC AGT(N-fluorescein)A, where N represents a spacer containing either a Cy3 or fluorescein group (SEQ ID NOS:50 or 51, respectively) and Probe 34-180-2: 5'-(N-TET)TTCCAGAGCC TAATTTGCCAGT-(N-fluorescein)A, where N represents a spacer containing either a TET or fluorescein group (SEQ ID NOS:52 or 53, respectively). Probe 34-180-1 (SEQ ID NO:50) has amino-modifiers on the two 5' end T residues and a Cy3 label on the 5' end, creating extra positive charges on the 5' end.

Probe 34-180-2 (SEQ ID NO:52) has a TET label on the 5' end, with no extra positive charges. The fluorescein label on the 3' end of probe 34-180-1 enables the visualization of the 3' cleaved products and uncleaved probes together on an acrylamide gel run in the standard direction (*i.e.*, with the DNA migrating toward the positive electrode). The 5' cleaved product of probe 34-180-1 has a net positive charge and will not migrate in the same direction as the uncleaved probe, and is thus visualized by resolution on a gel run in the opposite direction (*i.e.*; with this DNA migrating toward the negative electrode).

The cleavage reactions were conducted as follows. All conditions were performed in duplicate. Enzyme mixes for the *Pfu* FEN-1 and CLEAVASE A/G nucleases were assembled. Each 2 µl of the *Pfu* FEN-1 mix contained 100 ng of *Pfu* FEN-1 and 7.5 mM $MgCl_2$. Each 2 µl of the CLEAVASE A/G nuclease mix contained 26.5 ng of CLEAVASE A/G nuclease and 4.0 mM $MnCl_2$. Four master mixes containing buffer, M13mp18, and INVADER oligonucleotides were assembled. Each 7 µl of mix 1 contained 5 fmol M13mp18, 10 pmoles INVADER oligonucleotide 123 (SEQ ID NO:54) in 10 mM HEPES (pH 7.2). Each 7 µl of mix 2 contained 1 fmol M13mp18, 10 pmoles INVADER oligonucleotide 123 in 10 mM HEPES (pH 7.2). Each 7 µl of mix 3 contained 5 fmol M13mp18, 10 pmoles INVADER oligonucleotide 123 in 10 mM HEPES (pH 7.2), 250 mM KGlu. Each 7 µl of mix 4 contained 1 fmol M13mp18, 10 pmoles INVADER oligonucleotide 123 in 10 mM HEPES (pH 7.2), 250 mM KGlu. For every 7 µl of each mix, 10 pmoles of either probe 34-180-1 (SEQ ID NO:50) or probe 34-180-2 (SEQ IDNO:52) were added. The DNA solutions described above were covered with 10 µl of CHILLOUT evaporation barrier and brought to 65°C. The reactions made from mixes 1-2 were started by the addition of 2 µl of the *Pfu* FEN-1 mix, and the reactions made from mixes 3-4 were started by the addition of 2 µl of the CLEAVASE A/G nuclease mix. After 30 minutes at 65°C, the reactions were terminated by the addition of 8 µl of 95% formamide containing 10 mM EDTA. Samples were heated to 90°C for 1 minute immediately before electrophoresis through a 20% denaturing acrylamide gel (19:1 cross-linked) with 7 M urea, in a buffer containing 45 mM Tris-Borate (pH 8.3), 1.4 mM EDTA and a 20% native acrylamide gel (29:1 cross-linked) in a buffer containing 45 mM Tris-Borate (pH 8.3), 1.4 mM EDTA.

The products of the cleavage reactions were visualized following electrophoresis by the use of a Hitachi FMBIO fluorescence imager. The resulting images are shown in Fig. 21. Fig. 21A shows the denaturing gel, which was run in the standard electrophoresis direction, and Fig. 21B shows the native gel, which was run in the reverse direction. The reaction products produced by *Pfu* FEN-1 and CLEAVASE A/G nucleases are shown in lanes 1-8 and 9-16, respectively. The products from the 5 fmol M13mp18 and 1 fmol M13mp18 reactions are shown in lanes 1-4, 9-12 (5 fmol) and 5-8, 13-16 (1 fmol). Probe 34-180-1 is in lanes 1-2, 5-6, 9-10, 13-14 and probe 34-180-2 is in lanes 3-4, 7-8, 11-12, 15-16.

The fluorescein-labeled 3' end fragments from all cleavage reactions are shown in Fig. 21A, indicated by a "3"' mark at the left. The 3 nt 5' TET-labeled products are not visible in this Figure, while the 5' Cy3-labeled products are shown in Fig. 21B.

The 3' end bands in Fig. 21A can be used to compare the rates of cleavage by the different enzymes in the presence of the different 5' end labels. It can be seen from this band that regardless of the amount of target nucleic acid present, both the *Pfu* FEN-1 and the CLEAVASE A/G nucleases show more product from the 5' TET-labeled probe. With the *Pfu* FEN-1 nuclease this preference is modest, with only an approximately 25 to 40% increase in signal. In the case of the CLEAVASE A/G nuclease, however, there is a strong preference for the 5' TET label. Therefore, although when the charge reversal method is used to resolve the products, a substantial amount of product is observed from the CLEAVASE A/G nuclease-catalyzed reactions, the *Pfu* FEN-1 nuclease is a preferred enzyme for cleavage of Cy3-labeled probes.

EXAPMLE 4

Manual Coupling of the 5' Phosphoramidite (Positively Charged Phosphoramidite or Neutral Phosphoramidite) to Solid Support

This example demonstrates one means by which a phosphoramidite with a positive or neutral charge can be coupled to an oligonucleotide on a solid support. The

coupling method described below is provided by way of example and not by way of limitation; other coupling methods may also prove to be effective.

A ¼ inch plug of Pyrex Brand Fiber Glass Wool (Aldrich, Cat# Z 25,289-0) was tightly packed into a 2.5 ml gas-tight Hamilton syringe (VWR, Cat. # 90168) using first a pasteur piptte or like device to drive the glass wool to the bottom of the syringe, followed by compression with the syringe plunger. The plunger was removed and approximately 40 mg of dry Control Pore Glass (CPG) support, coupled with oligonucleotide sequence SEQ ID NO:55 (still protected with the dimethoxy trityl [DMT] moiety at the 5' end) was added to the syringe, on top of the packed glass wool. The amount of the CPG added varies with the batch of CPG synthesized, and is specifically dependent on the amount of oligonucleotide loaded onto the solid support. The plunger was reinserted and depressed to pack the CPG coupled DNA onto the glass wool. A 5-inch, 18 gauge Luer Lock needle was secured to the syringe, and all reagents were drawn into the reaction vessel (the syringe) via the needle. The plunger remained in the syringe for the rest of the procedure.

Once the plunger was reinserted, the CPG-oligonucleotide complex was washed 3 times with methylene chloride (stored over 3-angstrom pore size, activated, Molecular Sieves [Aldrich, Cat. # 20,858-2]) by drawing 1 ml into the syringe via the needle, inverting 3-5 times and ejecting the wash solution by depressing the plunger.

Reactions were then washed with 1 ml of deblock (dichloroacetic acid [a 15% solution in methylene chloride was special ordered from Glen Research] diluted to 3% in methylene chloride) to remove the DMT as described above. Washes were performed until the orange color generated by the free trityl groups was completely gone, with a maximum incubation time of 1 minute for all 3 washes.

After the final wash, the reactions were neutralized with three 1 ml washes of a 1:1 mixture of acetonitrile:pyridine, stored over calcium hydride. This was followed by 8, 2 ml washed with acetonitrile stored over calcium hydride. 1.5 ml of the appropriate phosphoramidite solution (either 50-100 mM of the positively charged or the neutral phosphoramidite in acetonitrile, stored over calcium hydride) and 1 ml of activator (0.25M 5-ethylthio-1H-tetrazole [Glen Research, Cat.# 30-3140) in anhydrous acetonitrile over activated Molecular Sieves) was drawn up into the syringe. The needle

was sealed using a silicone stopper (Aldrich, Cat.# Z16608-1) and rocked gently, by hand for 20 minutes at room temperature.

After the 20 minute incubation, the solution was ejected and six 1ml washes with acetonitrile stored over calcium hydride were done as described above. Two ml of oxidizer (0.02M iodine in tetrahydrofuran/pyridine/water [Glen Research, Cat.# 40-4330]) was drawn into the syringe, the needle was again sealed with a silicone stopper and the reaction was rocked gently at room temperature for 3 minutes. This was followed by 4, 1ml acetonitrile (stored over calcium hydride) washes and 2, 1ml acetonitrile:pyridine (1:1 mixture, stored over calcium hydride) washes. 1 ml of Cap B solution (10% n-methylimidazole in a solution of 8:1 tetrahydrofuran and pyrimidine [PE Biosystems]) and 1 ml of Cap A (THF/Acetic Anhydride, 9:1, PE Biosystems) were drawn into the syringe, the needle was capped and the reaction was rocked gently for 3 minutes at room temperature. This was followed by six 1 ml washes with acetonitrile:pyridine (1:1 mixture, stored over calcium hydride) and five 1 ml washes with methylene chloride stored over activated, Molecular Sieves.

For subsequent manual couplings, the above procedure can be repeated, starting with the deblock washes. For subsequent automated couplings, the support can be transferred to a synthesis column and attach to synthesizer. If the reaction is complete, the 5' dimethoxy trityl can be removed by washing with deblock, neutralizing with 3three 1 ml acetonitrile:pyrimidine washes, and eight 2 ml acetonitrile washes, as described above.

Deprotection Protocol:

The dried support (CPG) carrying the newly modified oligonucleotide was transferred to a 4ml glass vial (Wheaton, 224801) with a TEFLON-lined cap (Wheaton 240408). 1ml of concentrated ammonium hydroxide (EM Sciences AX 1303-13) was added and the reaction was incubated overnight at room temperature. The mixture was then Filter through a 0.2 μm TEFLON Acrodisc filter (Gelman, 4423T) using a 1ml disposable syringe (B-D, 309602), and finally dried to completion in a speedvac.

EXAMPLE 5

Synthesis of Positively Charged Phosphoramidite

1) Preparation of mono-DMT protected 4,4'-timethylene(*bis*(1-piperdine ethanol)):

10 grams (33.4 mmol) of 4,4'-timethylene(bis-(1-piperdine ethanol)) [Aldrich, Cat. # 12,122-3] and 1.46 ml (8.4mmol) of N-N-di-isopropylethylamine [Aldrich, Cat. # 38,764-9] were combined in a 250-ml round-bottom flask (such as ChemGlass, Cat.# CG-1506). A magnetic stir bar was added and stirring was initiated at medium speed. 2.84 grams (8.4mmol) of 4,4'-dimethoxytrityl chloride (Aldrich, Cat.# 10,001-3) was added as a solid, slowly (over the course of about 1 minute) with constant stirring. The flask was covered with a rubber septum and the reaction was incubated at room temperature with continued stirring, until complete, for about 1 hour.

The reaction was monitored by thin layer chromatography (EM Science 60F254 silica plates from VWR, Cat.# 5715-7) using standard methods known in the art until the starting material, 4,4'-dimethoxytrityl chloride, was no longer detected on the chromatography plate. The reaction products were then filtered and purified by column chromatography using a 4.5 by 25 cm glass chromatography column (with glass frit and TEFLON stopcock) and 70-230 mesh, 60 angstrom silica gel (Aldrich, Cat.# 28,862-4). The running solvent was a solution of 5% methanol, 5% triethylamine and 90% methylene chloride. Chromatography was performed by standard methods known in the art. The product was a yellow oil, with a yield of approximately 4.8 grams (95%) with an Rf value of 0.55 as determined by TLC. TLC was performed using EM Science $60F_{254}$ silica plates (VWR, Cat.# 5715-7), in a running buffer of 5% triethylamine/95% dioxane.

2) Preparation of phosphoramidite:

1.3 grams (2.2mmol) of mono-DMT protected 4,4'-timethylene(bis-(1-pipirdine ethanol)) synthesized in the above reaction was co-evaporated in a 250 ml round bottom flask, three times with 20 ml of acetonitrile. A Büchi Rotovapor with dry ice/alcohol condenser, (Büchi, model number R-114) was used for the evaporation, and the mixture was dried to completion for each co-evaporation.

The dry product was then dissolved in 12 ml of methylene chloride followed by an addition of 0.85 ml (2.7mmol) of 2-cyanoethyl tetraisopropyl phosphorodiamidite (Aldrich, Cat.# 30,599-5). 122mg (1.7mmol/4ml) of tetrazole dissolved in 3 ml dry acetonitrile was added with vigorous swirling, and the reaction vessel was secured in a cork ring, taped to a vortexer and vortexed at medium speed, at room temperature, for 1.5 hours. The reaction was monitored by TLC and was complete when mono-DMT protected 4,4'-timethylene(bis(1-piperdine ethanol)) was no longer visible by TLC.

25 ml of methylene chloride were added to increase the volume, and the entire reaction was transferred to a 100ml separatory funnel. An equal volume (approximately 40 ml) of a 5% sodium bicarbonate:1% triethylamine solution was added, the mixture was shaken for 15 seconds and allowed to equilibrate. The lower, organic phase was drained from the funnel and retained. The upper aqueous phase was discarded, the organic phase was transferred back to the separatory funnel and the wash was repeated for a total of three sodium bicarbonate/triethylamine washes.

The organic phase was transferred to an Ehrlenmeyer flask and solid magnesium sulfate (approximately 20g) was slowly added, with swirling, until no clumping of the solids was detected. The magnesium sulfate was filtered via a Büchner filter funnel with ground glass adaptor (Chemglass, Cat.# CG-1406) and the solution was concentrated and co-evaporated twice with 20 ml of acentonitrile on a Büchi Rotovapor in a tared, round-bottom flask. The amount of dry product was determined by mass, and then re-dissolved in acetonitrile to a final concentration of approximately 150-200mg/ml. Several granules of calcium hydride were added. The dissolved product was then dispensed (2 ml/bottle) into amber glass vials (Wheaton, Cat. # 224754) and dried, first via a water aspirator until the product appears as an extremely viscous oil, and then overnight under vacuum in a glass dessicator (VWR) containing phosphorous pentoxide (Aldrich, Cat. # 29822-0) and DRIERITE (VWR, Cat. # 22891-040). The yield was approximately 1.6 grams (92.1%) with an Rf value of 0.7 as determined by TLC. TLC was performed using pre-run EM Science $60F_{254}$ silica plates (VWR, Cat.# 5715-7), in a running buffer of 5% triehtylamine/95% dioxane.

EXAMPLE 6

Synthesis of Neutral Phosphoramidite

1) Synthesis of mono-DMT protected N-methyldithanolamine:

8.3 grams (70.0 mmol) of N-methyldiethanolamine, 2.2 ml (12.6 mmol) of di-isopropyl ethylamine and 100 ml of acetonitrile were combined in a 250-ml round-bottom flask (such as ChemGlass, Cat.# CG-1506). A magnetic stir bar was added and stirring was initiated at medium speed. 4 grams (11.8 mmol) 4,4'-dimethoxytrityl chloride (Aldrich, Cat.# 10,001-3) was added as a solid, slowly (over the course of about 1 minute) with constant stirring. The flask was covered and the reaction was incubated at room temperature with continued stirring, until complete, for about 1 hour. The reaction was monitored by thin layer chromatography (EM Science $60F_{254}$ silica plates from VWR, Cat.# 5715-7) using standard methods known in the art. The reaction is complete when the starting material, N-methyldiethanolamine is no longer detected on the chromatography plate.

After the 1 hour incubation, the reaction products were concentrated using the Büchi Rotovapor, and then dissolved in 50 ml of methylene chloride. The dissolved product was transferred to a 250 ml glass separatory funnel and washed 3 times with 50 ml of 5% sodium bicarbonate and once with saturated sodium chloride, as described above.

The reaction products were then filtered and purified by column chromatography using a 4.5 X 25 cm glass chromatography column (with glass frit and TEFLON stopcock) and 70-230 mesh, 60 angstrom silica gel (Aldrich, Cat.# 28,862-4). The running solvent was a solution of 5% methanol, 5% triethylamine and 90% methylene chloride. Chromatography was performed by standard methods known in the art. The product was a yellow oil, with a yield of approximately 4.8 grams (95%), with an R_f value of 0.55 as determined by TLC. TLC was performed using pre-run EM Science $60F_{254}$ silica plates (VWR, Cat.# 5715-7), in a running buffer of 5% triehtylamine/95% dioxane.

2) preparation of phosphoramidite:

1.3 grams (3.2mmol) of mono-DMT protected N-methyldiethanolamine, synthesized in the above reaction, was co-evaporated in a 250 ml round bottom flask, three times with 20 ml of acetonitrile (ACN). A dry ice/alcohol, Büchi Rotovapor, (Büchi, model number R-114) was used for the evaporation, and the mixture was dried to completion for each co-evaporation.

The dry product was then dissolved in 12.6 ml of methylene chloride followed by and addition of 1.2 ml (3.8 mmol) of 2-cyanoethyl tetraisopropyl phosphorodiamitide (Aldrich, Cat.# 30,599-5). 173mg (2.5mmol/4ml) of tetrazole/acetonitrile was added with vigorous swirling, and the reaction vessel was secured in a cork ring, taped to a vortex and vortexed at medium speed, room temperature, for 3 hours. 25 ml of methylene chloride were added to increase the volume, and the entire reaction was transferred to a 100 ml separatory funnel. An equal volume (approximately 40 ml) of a 5% sodium bicarbonate:1% triethylamine solution was added, the mixture was shaken for 3-5 seconds and allowed to equilibrate, and the lower, organic phase was drained from the funnel and saved. The upper aqueous phase was discarded, the organic phase was transferred back to the separatory funnel and the wash was repeated, for a total of three sodium bicarbonate/triethylamine washes. The organic phase was transferred to an Ehrlenmeyer flask and solid magnesium sulfate (approximately 20g) was slowly added, with swirling, until no clumping of the solids was detected. The magnesium sulfate was filtered out via a Büchner filter funnel with ground glass adaptor (Chemglass, Cat.# CG-1406), and the solution was concentrated and co-evaporated twice with 20 ml of acentonitrile in a Büchi Rotovapor in a tared, round-bottom flask. The amount of dry product was determined by mass, and as then re-dissolved in acetonitrile (and several granules of calcium hydride) to a final concentration of approximately 150-200mg/ml. The dissolved product was then aliquoted (2 ml/bottle) into amber glass bottles (Wheaton) and dried, first via a water aspirator until the product appears as an extremely viscous oil, then overnight under vacuum in a glass dessicator (VWR) containing phosphorous pentoxide (Aldrich) and DRIERITE (VWR). The yield was approximately 1.9 grams (97.0%) with an Rf value of 0.8 as determined by TLC. TLC was performed

using pre-run EM Science $60F_{254}$ silica plates (VWR, Cat.# 5715-7), in a running buffer of 5% triehtylamine/95% dioxane.

EXAMPLE 7

Synthesis of the 1,6 Hexanediol H-Phosphonate

1) Synthesis of the DMT protected 1,6-Hexanediol

Three grams (25mmol) of 1,6-hexanediol (Aldrich, Cat.24,011-7) was dissolved in 120mL of anhydrous tetrahydrofuran (THF) (Aldrich, Cat.# 18,656-2). 1.5 mL (1.1g, 88mmol) of di-isopropylethylamine (Aldrich, Cat.# 38,764-9) were added, and the resulting mixture (protected from moisture) was stirred at room temperature for 15 minutes. Three grams (9 mmol) of Dimethoxytrityl Chloride (DMTCl) was then added, and the solution was incubated, with stirring for two hours at room temperature. The resulting mixture was concentrated under reduced pressure via a Büchi Rotovapor (Büchi, model R-114), and the concentrated material was filtered and purified via column chromatography using silica gel column (70-230 mesh) / Hexane: Ethyl Acetate 1:1 by standard methods known in the art. Fractions containing isolated material (as determined by TLC; $R_f = 0.3$) were combined and concentrated. The yield was 77% (2.9g; 7mmol).

2) Synthesis of the DMT-1,6-Hexanediol H-phosphonate

All reactions described below were performed under nitrogen in a system protected from moisture.

a) Synthesis of the Phosphorus Triimidazolide (PIm_3)

4.3 mL (5.9g; 43mmol) of Phosphorus trichloride (PCl_3, Aldrich, Cat.#31,011-5) was dissolved in 100mL of anhydrous THF at 0° C with gentle stirring. The temperature was held at 0°C, and stirring was continued while, over a period of 10 minutes, 18.8mL (18g, 129mmol) of Trimethylsilylchloride (Me_3Si-Cl, Aldrich, Cat.#C7,285-4) dissolved in 40mL of anhydrous THF was added to the reaction. After the addition of Me_3Si-Cl, the reaction mixture was incubated at 0°C for 30 minutes with continued stirring, and then at room temperature for 30 minutes with continued stirring. Finally, the reaction mixture

was concentrated under reduced pressure, protected from moisture, to 75% of its original volume.

b) Synthesis of H-Phosphonate

5.9g (14mmol) of the DMT-protected 1,6-hexanediol synthesized above was dissolved in 10mL of anhydrous acetonitrile, and was then added slowly (over a period of about 5 minutes, with constant stirring) at room temperature, to the phosphorus triimidazolide (PIm_3) solution. The reaction was incubated at room temperature with stirring for 4 hours, and then transferred to a separatory funnel containing 100 ml of water, 50 g of ice, 20ml of Triethylamine and 50ml of methylene chloride. The organic and aqueous phases were allowed to separate, and the organic (lower) fraction was isolated. The extraction was repeated until no DMT-containing material was present in the organic fraction as determined by TLC, described previously. Combined organic fractions were dried over magnesium sulfate for 1hr, followed by concentration under reduced pressure. The concentrated product was purified by column chromatography using Silica gel 70-230 mesh, methylene chloride / methanol 10% / Triethylamine 5% (R_f =0.5).

Product containing fractions were combined and concentrated. Yield: 5.8g (61%). The final concentrated product was then co-evaporated 5 times with 50 ml of anhydrous Acetonitrile, dried under high vacuum for 18 hours and dissolved in 18mL of Pyridine/Acetonitrile 1:1. Activated Molecular sieves (3 angstrom) were added.

EXAMPLE 8

Manual introduction of modifications into CRE Probes using H-phosphonate chemistry

A 2.5 ml gas-tight Hamilton syringe (VWR, Cat.#90168) was loaded (as detailed in Example 4) with 1 μmol CPG support (DMT on) coupled with a DNA CRE probe (for example, SEQ ID NO:55).

To remove the DMT, the CPG/oligonucleotide complex was washed twice (as described in Example 4) with 1 ml of methylene dichloride, then washed for 1 minute

with 5 ml of 3% dichloroacetic acid in methylene dichloride. The reaction was then washed 10 times with 1 ml of anhydrous acetonitrile/pyridine 1:1. After the final wash, one of 5 different H-phosphonate moieties (the 1,6 hexanediol H-phosphonate synthesized in Example 7; dA-H-Phosphonate, dC-H-Phosphonate, dG-H-Phosphonate, or dT-H-Phosphonate [Glen Research, Cat.# 10-1200-05, 10-1210-05, 10-1220-05, 10-1230-05]) was added as follows. 1 ml of H-phosphonate solution (concentration: 50 – 150μmol/mL) and 1mL of the trimethylacetyl chloride solution in anhydrous acetonitrile/pyridine 1:1 (concentration: 100 - 250μmol/mL) were drawn into the syringe, the needle was sealed and the reaction was incubated at room temperature with gentle shaking for 5-10 minutes. The syringe contents were expelled, and 6, 1ml acetonitrile/pyridine 1:1 washes were done. After the last wash, 0.1-0.2g of a primary or secondary amine (for example N,N-dimethylethylenediamine, Aldrich, Cat.#D15,780-5) in 1mL of anhydrous pyridine, followed by 0.5 mL of anhydrous carbon tetrachloride were drawn into the syringe and incubated at room temperature, with gentle shaking for 5-15 minutes. The syringe contents were expelled, and six 1 ml anhydrous acetonitrile/pyridine 1:1 washes were done. This was followed by six 1 ml methylene chloride washes; a 1 minute wash with 5 ml 3% dichloroacetic acid/methylene dichloride; ten 1 ml washes with anhydrous acetonitrile/pyridine 1:1 and six 1 ml washes with methylene chloride.

The dried support (CPG) was transferred to a 4ml glass vial (Wheaton, 224801) with a TEFLON-lined cap (Wheaton 240408). 1ml of concentrated ammonium hydroxide (EM Sciences AX 1303-13) was added and the reaction was incubated for 12 hours at 55°C. After the cleavage and deprotection was completed, the product containing ammonia solution was concentrated under reduced pressure and subjected to ion exchange HPLC or reverse phase HPLC purification.

For all HPLC purifications, the Hitachi HPLC (Interface model# D-7000; pump model# 7100; diode array detector model# L-7455) system, and standard methods known in the art were used. The specific conditions used for the Reverse Phase HPLC purification were: C-18 Dionex analytical column (4.6x250mm) with a flow rate of 1 ml/min, starting with 100% buffer A (0.1M TEAA) and 0% buffer B (acetonitrile), and transitioning to buffer B at a rate of 1% buffer B per minute. Fractions were collected

and analyzed via mass spectrometry by methods known in the art, to identify the complete product.

The specific conditions used for the ion exchange HPLC purification were: Amersham Pharmacia Biotech HR 10/10 15Q IE column (10X100mm) with a flow rate of 5 ml/min. Buffer A (20mM sodium perchlorate, 20 mM sodium acetate, 10% acetonitrile, pH 7.35) and Buffer B (600 mM sodium perchlorate, 600 mM sodium acetate, 10% acetonitrile, pH 7.35) were used in a gradient beginning and ending at 5%A/95%B, with a gradient increase of approximately 65%B per minute. Fractions were collected and analyzed by mass spectrometry by methods known in the art, to identify the desired product.

EXAMPLE 9

Effect of tag modifications on the INVADER Assay reaction

In this example, oligonucleotide probes containing positively charged tags at their 5' ends were tested in INVADER assay reactions, and the reaction turnover rates using two, differently modified probe oligonucleotides were compared. Here, turnover rate is defined as the number of cleavage events per target per unit time. The turnover rates were determined as described in (Lyamichev, *et al.*, Biochemistry 39:9523 [2000]).

The first oligonucleotide probe, 5'-Cy3-AminoT-AminoT-ACG CCA CCA GCT-3' (SEQ ID NO:56, termed 203-85-5), utilized AminoT modifications such as those described in Example 2.

The second oligonucleotide probe, 5'-V-(Hex)-Cy3-CGC TGT CTC GCT-3' (SEQ ID NO:57, termed 490-52), was synthesized using the H-phosphonate modification V-(Hex), depicted in Figure 11. The INVADER-directed cleavage of probes 203-85-5 and 490-52 was designed to release net positively charged Cy3-labeled products 5'-Cy3-AminoT-AminoT-3' and 5'-V-(Hex)-C-3', respectively. The first product is generated by enzymatic cleavage after AminoT, whereas the second product is produced by the cleavage after a natural base C.

The INVADER oligonucleotide 5'-GCT CAA GGC ACT CTT GCC C-3' (SEQ ID NO:58, termed 203-85-4) and the target oligonucleotide 5'-ATG ACT GAA TAT

AAA CTT GTG GTA GTT GGA GCT GGT GGC GTA GGC AAG AGT GCC TTG ACG ATA-3' (SEQ ID NO:59, termed 203-85-3) used with the probe 203-85-5 were synthesized using phosphoramidite reagents obtained from Glen Research and standard phosphoramidite chemistries known in the art. The underlined nucleotides denote 2'-O-methyl modifications. The INVADER and target oligonucleotides used with the probe 490-52 were combined into the single molecule 5'-biotin-TTT TTT TTT AAT TAG GCT CTG GAA AGA CGC TCG TGA AAC GAG CGT-3' (SEQ ID NO:60, termed IT5). All oligonucleotides were gel purified and quantitated as described (Lyamichev, *et al.*, *supra*).

The INVADER assay reactions utilizing the AminoT-modified probe 203-85-5 were performed as follows: 10 µl reactions were prepared and contained (final concentrations): 2 µM amino modified probe (203-85-5), 1 µM INVADER oligonucleotide 203-85-4 (SEQ ID NO: 58), 1 nM target oligonucleotide 203-85-3 (SEQ ID NO:59), 32 nM AfuFEN1 CLEAVASE enzyme, 10 mM MOPS, pH 7.5, and 4 mM $MgCl_2$.

The INVADER reactions utilizing probe 490-52 (2 µM) were prepared as above, except 1 nM of the IT5 oligonucleotide (SEQ ID NO:60) was used, and served as both the INVADER oligonucleotide and the target oligonucleotide.

The reactions were assembled on ice in 200µl thin wall PCR tubes (Dot Scientific, Cat.#620-PCR), overlaid with 10 µl of Chill-out liquid wax (MJ Research) and transferred to a Mastercycler heating block (Eppendorf, Cat.# 5331 000.045). The reactions were incubated for 60 minutes at 55.3, 57.7, 60.5, 63.4, 66.2, and 68.7°C using a temperature gradient of 62±10°C (controlled by the heating block). The reactions were stopped after 1 hour with the addition of 10 µL of 95% formamide containing 20 mM EDTA and 0.02% methyl violet.

One microliter aliquots of each reaction were loaded onto each of two 200x200x1 mm slabs of 15% denaturing polyacrylamide gel (crosslinked 19:1) with 7 M urea in a buffer containing 45 mM Tris borate, pH 8.3 and 1 mM EDTA. An electric field of 20 watts was applied for 30 minutes with the positive electrode connected either to the top buffer reservoir (reverse orientation) or bottom reservoir (normal orientation). The net positively charged products generated in the course of the INVADER reactions were

detected by gel electrophoresis in the reverse orientation and the uncleaved probes of the same samples were analyzed by separation in the normal orientation. The intensities of bands corresponding to the products and uncleaved probes were measured using FMBIO-100 fluorescence imager (Hitachi, Alameda, CA) equipped with 532-nm laser and 585-nm filter at 10% sensitivity level.

The measured turnover rates for probes 203-85-5 (SEQ ID NO:56) and 490-52,(SEQ ID NO:57) as a function of temperature are shown in Fig. 22. The probe 490-52 which was synthesized using H-phosphonate chemistry to introduce the modification V-(Hex), has approximately 10-fold greater turnover rate than the AminoT modified probe 203-85-5.

EXAMPLE 10

Detection of Specific Cleavage Products by Charge Reversal

This example demonstrates that a CLEAVASE enzyme that recognizes cleavage structures containing RNA targets (CLEAVASE TthAKK) also recognizes and cleaves structures containing RNA targets and the above-described positively charged probe oligonucleotides. In this example, 5 different, modified probe oligonucleotides were used in an INVADER reaction to detect human MCP1 *in vitro* transcripts. Each probe oligonucleotide was designed to release a labeled product with a net positive charge such that the cleavage products could be detected using charge reversal methods.

The five different, 5'-end modified, Cy3-labeled probe oligonucleotides tested were: 5'- V-(HEX)-Cy3 -CTTCGGAGTTTGGG-NH_2-3' (SEQ ID NO:61; termed "oligo P1"), 5'- V-(dA)-Cy3 -CTTCGGAGTTTGGG-NH_2-3' (SEQ ID NO:62; termed "oligo P2"), 5'- V-(dC)-Cy3 -CTTCGGAGTTTGGG-NH_2-3' (SEQ ID NO:63; termed "oligo P3"), 5'- V-(dG)-Cy3 -CTTCGGAGTTTGGG-NH_2-3' (SEQ ID NO:64; termed "oligo P4"), and 5'- V-(dT)-Cy3 -CTTCGGAGTTTGGG-NH_2-3' (SEQ ID NO:65; termed "oligo P5") (Fig. 23). The 5' modifications were synthesized as described previously, and all 5 of the above oligonucleotides and the INVADER oligonucleotide, Inv1 5'-GGGTTGTGGAGTGAGTGTTCAAGTA-3'(SEQ ID NO:66) were chemically

synthesized on a DNA synthesizer (ABI 391) using standard phosphoramidite chemistries and reagents obtained from Glen Research (Sterling, VA).

All probe oligonucleotides were purified by Anion exchange HPLC. There was one major and one or more minor peaks observed with this purification method. The material from the major (first) peak was used in all experiments described below.

In vitro transcripts were synthesized as follows. The human Ubiquitin cDNA was isolated from a first-strand human liver cDNA library (Clontech Cat #7407-1) by PCR using a universal 5' primer (AP1, 5'CCATCCTAATACGACTCACTATAGGGC-3', SEQ ID NO:67) provided with the library and a Ubiquitin-specific 3' primer (5'-CTCATACAGTTACTTGTCTTC-3', SEQ ID NO:68). PCR reactions were performed with an error-correcting polymerase mixture from Clontech (Cat # 8417-1) according to manufacturer's instructions. The expected size of the PCR products was 500 bases. PCR products were gel purified on 1% agarose gel run in 0.5X TBE. The gel was Stained in 10μg/ml ethidium bromide, visualized under UV light, the appropriately sized band was excised and the DNA recovered with a QIAquick Gel Extraction Kit (Qiagen Cat #28706). The gel-purified fragment was then cloned into the pCR2.1-TOPO cloning vector (Invitrogen, Cat. # K4500-01) by methods known in the art. Positive clones were selected and insert identity was confirmed by DNA sequencing. The positive plasmids were transformed into TOP10 cells (Invitrogen). Cells were grown and plasmid isolated by methods well known in the art of molecular biology. The same 5' and 3' primers used above were then used in PCR reactions to generate templates for use in *in vitro* transcription reactions. *In vitro* transcriptions were done performed using the Ambion T7 MEGAshortscript RNA Transcription Kit (Ambion, Cat.# 1354) according to the manufacturer's instructions. The resulting human ubiquitin transcript is SEQ ID NO:69. Note that the use of the AP1 5' primer includes the T7 RNA polymerase promoter, which is necessary for the generation of *in vitro* transcripts. All transcripts used in the following reactions contained tRNA (Sigma) at 20ng/ μl as carrier.

HMCP1 i*n vitro* transcripts were synthesized as follows. The human Monocyte Chemoattractant Protein-1 (hMCP-1) cDNA was obtained from 10 ug/ml Con-A (concanavalin-A) and PHA (phytohemagglutinin) stimulated human PMBC's (Peripheral Blood Mononuclear Cells) total RNA. Total RNA was isolated from $1x10^7$ cells with

TRIzol®Reagent (Gibco BRL Cat #15596) according to the manufacturing protocol. 500 ng of total RNA was used for reverse transcription using the GeneAmp RNA PCR kit (Perkin Elmer cat #N808-0017) for the generation of the cDNA. This RT-PCR was performed using a gene specific 5' primer that also contained the T7 RNA polymerase promoter site (5'-GGAATACGACTCACTATAGGGAAAGTCTCTGCCGCCCTTCTGTGCCTGCTGC-3', SEQ ID NO:70) and a 3' hMCP-specific primer (5'-AATAGTTACAAAATATTCATTTCCACAATAA-3', SEQ ID NO:71). The 665 base fragment was re-amplified using the same PCR primers and Taq DNA Polymerase (Perkin Elmer Cat. #N808-0152). The fragment was column purified using the Wizard® PCR Preps DNA Purification System (Promega Cat # A7170) and quantitated by $O.D._{260}$ measurement. *In vitro* transcription was performed using 600 ng of the purified PCR product in the Ambion T7 MEGAshortscript RNA Transcription Kit (Ambion Cat #1354) according to the manufacturer's protocol. The hMCP *in vitro* transcript generated (SEQ ID NO:72) was 647 nt long.

The solution of the *in vitro* transcript was mixed with an equal volume of loading dye (95% Formamide, 10mM EDTA, Methyl violet dye), heat denatured at 90°C for 3 minutes and then loaded on a 6% denaturing (19:1 cross-linked) with 7 M urea acrylamide gel run in 0.5X TBE. After the electrophoresis, one of the glass plates was removed and the gel was covered with plastic wrap. The gel then was placed wrap-side-down on the TLC (DC Fertigplatten Kieselgel 40 F_{254} Merck, Art 5634) plate and the other glass plate was removed. The RNA bands were visualized in the dark room by shining a hand–held UV light source (254nM; short wave) on the surface of the gel. The nucleic acid will appear as dark bands while the TLC plate will appear green. The bands corresponding to the RNA were excised with a razor blade and eluted in TE (10 mM Tris, 0.1 mM EDTA) containing 0.3 M sodium acetate at 37°C for 4 hours. The *in vitro* transcript was ethanol precipitated at -20°C over night (alternatively, precipitation at -70° C for 1hour is also sufficient) and pelleted at 14,000 rpm for 30 min at 4°C. The pelleted nucleic acid was then washed with 70 % ethanol and spun again for 5 minutes. After the ethanol was discarded, the pelleted nucleic acid was dried under vacuum and resuspended in RNase-free H_2O (USB Cat #US70783). The concentration of the *in vitro* transcript

was determined by OD260. All dilutions of the *in vitro* transcript used in the reactions were prepared in 20ng/μl of yeast tRNA (Sigma Cat # R5636).

Five sets of reactions were done, one for each different probe oligonucleotide. A negative (no-target) control containing 100 ng of yeast tRNA was performed for each reaction set. Each 10 μl reaction was prepared at room temperature as follows. Five different master mixes were prepared, one for each probe. Each mix comprised (final concentration): 10 mM MOPS, pH 7.5, 100 mM KCl, 0.05% Tween, and 0.05 % Nonidet NP40, 12.5 mM $MgSO_4$, 5 pmoles of INVADER oligonucleotide (SEQ ID NO:66) and 20ng of CLEAVASE TthAKK enzyme. Finally, 10 pmoles of one of the probes (SEQ ID NOS:61, 62, 63, 64 or 65) were added for a final volume of 10 μl per reaction/per master mix. The master mixes were vortexed briefly and 5 μl of each was transferred to the appropriate reaction vessel (200 μl thin wall PCR tubes, Dot Scientific, Cat. #620-PCR), followed by the addition of 5 μl (containing 0, 0.1, 1 or10 fmoles) of human MCP1 *in vitro* transcript. 100 ng of yeast tRNA (Sigma) was used as a negative control. Samples were pipetted up and down 3 times to mix. The samples were then overlaid with 10μl colored Chill out 14 liquid wax (MJ Research) to prevent evaporation and incubated at 63°C for 60 min. Reactions were terminated by the addition of 50 μl of 95% formamide containing 10 mM EDTA.

Samples were run on a 15% denaturing acrylamide gel (19:1 cross-linked) with 7 M urea, in a buffer containing 45 mM Tris-Borate (pH 8.3), 1mM EDTA. The gel was pre-run, with the electrodes in the normal orientation prior to loading. The samples were heated to 90°C for 1 minute immediately before loading, and 2 μl were loaded per well. An electric field of 20 watts was applied for 30 minutes with the electrodes in the normal orientation. The products were visualized following electrophoresis with a Hitachi FMBIO fluorescence imager with 585-nM filter at 20 % sensitivity. The gel was then replaced on the running apparatus, and fresh buffer was added to the reservoirs. The electrodes were then placed in the reverse orientation, the gel was pre-run and loaded as above. The gel was run for 1 hour in the reverse orientation, and products were visualized as above. The resulting images are shown in Fig. 24. Fig. 24A shows the denaturing gel, which was run in the standard electrophoresis direction, and Fig. 24B shows the denaturing gel, which was run in the reverse direction. Probe V-(HEX) panel

A; probe V-(dA) panel B; probe V-(dC) panel C; probe V-(dG) panel D; and probe V-(dT) panel E.

EXAMPLE 11

Effects Of a 5' Positive Charge on Cleavage Rate using CLEAVASE TthAKK Enzyme

The previous example demonstrated the ability of the CLEAVASE TthAKK enzyme to recognize and cleave a cleavage structure containing an RNA target and a positively charged probe oligonucleotide. This example tests the effect of the positively charged probes on cleavage rates.

All 5 of the positively charged probe oligonucleotides described in Example 10 were tested against a 5' fluorescein labeled "control" probe oligonucleotide (SEQ ID NO:73; 5' fluorescein phosphoramidite from Glen Research). Both the positively charged and the control probe were designed to detect the same sequence, so are identical in the analyte specific region. The difference between the fluorescein labeled and the CRE-V labeled probes include the charge difference at the 5' end, and the length of the cleaved products, or 5' flap. The 5' flap of the positively charged probes is 1 base, while the control probe yields a 3 base, 5' flap.

Reactions were performed as described in Example 10, using the hMCP1 *in vitro* transcripts as target. Only one target level was used to test the cleavage rate for each probe oligonucleotide. Each reaction received either 1 fmole of the hMCP1 *in vitro* transcript with 100 ng of yeast tRNA as carrier; 100 ng of yeast tRNA also served as a negative control. Reactions containing target were done in quadruplicate, while the tRNA control reactions were done singly.

Turnover rates were determined as described in Lyamichev, *et al.*, *supra*, and are shown graphically in Fig. 25. The rates ranged from 2-to 9 cleavage events/target/minute with P3 (SEQ ID NO:63) showing the highest rate among the positively charged probes. The average cleavage rate of the fluorescein labeled probe was 12 cleavage events/target/minute.

EXAMPLE 12

Examination of the Rate of Background Accumulation With 5' Positively Charged Probe Oligonucleotides

A key advantage to using positively charged probe oligonucleotides is the ability to completely separate signal (e.g., the single base flap carrying the positively charged signal molecule) from any other aberrant reaction products or uncleaved probes using simple, reverse polarity gel electrophoresis, as described and detailed in the above examples. This experiment confirms that background cleavage products (aberrant cleavage, or thermodegradation products) will not migrate in the reverse polarity gel, even if the reaction is incubated with large amounts of target for an extended period of time, allowing for greater certainty and simplicity in data interpretation.

The probe oligonucleotide used was P2 (described in Experimental Example 10, SEQ ID NO:62) and the INVADER oligonucleotide used was Inv1 (SEQ ID NO:66), also described in Example 10. The reaction conditions and gel based separation method were performed as described in 10. Reactions were performed with 0 (100ng/5 µl of tRNA as a negative control; background estimate), 0.01, 0.1 and 1 fmole of hMCP1 *in vitro* transcript in a 10µl reaction volume. Reactions were assembled as described in Example 10, and incubated for 1, 2, 4, 8 and 24 hours at 63°C. Reaction products were separated in normal or reverse polarity gels, as described in Example 10, and were analyzed based on the intensities from the Hitachi FMBIO scanner images and software, also described in Example 10. The results are shown graphically in Fig. 26. Fig. 26A represents the results of the denaturing gel, which was run in the standard electrophoresis direction, and Fig. 26B represents the results of the denaturing gel, which was run in the reverse direction.

EXAMPLE 13

Detection of an RNA target using multiple, positively charged probes.

The previous experiments have demonstrated that the positively charged probes cleaved in a structure specific manner by the CLEAVASE enzyme, can be used to detect RNA targets, and, in certain detection platforms, can be analyzed such that the signal to background ratio is superior to "normal," negatively charged probe oligonucleotides.

The present experiment demonstrates that the cleavage products of different, 5' positively charged probes can be distinguished (based on the different mass to charge ratios), even when used in the same reaction.

The oligonucleotides used in this experiment, the reaction conditions, gel-based separation and the analysis were conducted as described in Example 10, except that 2 pmoles of each of 4 different probes [P1, P2, P4, and P5] were used, and the target levels were 0 (100 ng of tRNA only), 0.1, 1 and 10 fmoles of hMCP1 *in vitro* transcript. Two µl of each reaction was loaded on the gel in reverse polarity and separated as described.

The resulting image is shown in Figure 27. All cleavage products have a net positive charge. The mobility of the cleaved products from probe oligonucleotides P1, P2 and P4 were easily separated on the gel due to the differences in size (molecular weight) between them. In contrast, the cleaved products from the P5 probe oligonucleotide were barely distinguishable from the P4 products; the size and charge of these products are very similar. This demonstrates that a preferred, multiplex embodiment utilizes probes whose cleaved products can be easily distinguished in the detection system of choice.

EXAMPLE 14

Human MCP1 and Human Ubiquitin *in vitro* Transcript Detection in a Cascade Reaction with Positively Charged Tags

In this example, a two-step, sequential invasive cleavage reaction is used to detect both hMCP1 and hUbiquitin *in vitro* transcripts, in a true, multiplex reaction (both targets are detected in the same reaction). The positively charged probes (termed reporter oligonucleotides, or reporter-labeled oligonucleotides in this example) are used in the second step of the sequential invasive cleavage reaction, as shown in Fig. 28A and B. The added amplification provided by the cascading INVADER scheme yields greater sensitivity and lower limits of detection, important if target levels are limiting.

The mechanism of the sequential invasive cleavage reaction is as follows. The primary INVADER and probe oligonucleotides (those which hybridize to the target) are unlabeled and, when hybridized to the appropriate target sequence, form the overlapping

structure recognized by the CLEAVASE enzyme (Fig. 28A). The enzyme cuts the structure and frees the 5' flap. The flap then acts as an INVADER oligo for the secondary reaction. The secondary reaction comprises 3 different oligonucleotides: 1) a flap-reporter bridging oligonucleotide that has adjacent regions complementary to both the 5' flap and the reporter- labeled, secondary probe oligonucleotide; 2) a reporter-labeled, secondary oligonucleotide, complementary to a portion of the bridging oligonucleotide, and 3) the INVADER oligonucleotide, which is the 5' flap from the primary reaction, and which is complementary to a portion of the bridging oligonucleotide. When the overlapping structure forms in the secondary reaction, the enzyme cleaves the 5' flap from the reporter-labeled oligonucleotide, generating detectable signal with a positive charge.

In the secondary reaction, the 5'-flaps of the uncleaved probe molecules can compete with the released 5'-flaps for hybridization to the flap-reporter bridging oligo, thus decreasing signal generation in the secondary reaction. To avoid this competition, the uncleaved probe is sequestered after the primary incubation by the addition of a complementary oligonucleotide called an "ARRESTOR oligonucleotide." The ARRESTOR oligonucleotide is fully complementary to the target-specific region of the probe, and partially extends into the 5'-flap region; thus, it does not interfere with the binding of the 5'-flap to the flap-reporter bridging oligonucleotide. ARRESTOR oligonucleotides thus promote more effective signal generation in the secondary reaction by preventing interactions between uncleaved probes and flap-reporter binding oligonucleotides. All of the bases of the ARRESTOR oligonucleotide are 2' O-methyl-modified, making the ARRESTOR oligonucleotide resistant to cleavage by the CLEAVASE enzyme.

The tag used for the hMCP1 secondary, reporter probe oligonucleotide was 5' V(dC)-Cy3 (Fig. 28A), while the hUbiquitin secondary, reporter probe oligonucleotide incorporated the 5' V(dG)-Cy3 tag (Fig. 28B). These tags were chosen since, as demonstrated in Example 10 and shown in Figure 24 they are easily separated and identified due to the difference in mass-to-charge ratio between them. The oligonucleotides used for the detection of Human MCP1 *in vitro* transcripts were: the

primary probe oligonucleotide 5'-CCGTCACGCCTCCTTCGGAGTTTGGG-NH_2-3' (SEQ ID NO:74), the primary INVADER oligonucleotide Inv1 (SEQ ID NO:66), the arrestor oligonucleotide 5'AACCCAAACTCCGAAGGAGGCGTG-NH_2-3' (SEQ ID NO:75), the flap-reporter bridging oligonucleotide 5' GCGCAGTGAGAATGAGGAGGCGTGACGGT-NH_2-3' (SEQ ID NO:76), and the reporter-labeled secondary probe oligonucleotide 5'-V(dC)--Cy3 CTCATTCTCAGTGCG-3' (SEQ ID NO:77). The underlined bases denote 2'-O-methyl modifications. The oligonucleotides used for the detection of Human Ubiquitin *in vitro* transcripts were: the primary probe oligonucleotide 5'-AACGAGGCGCACCTTTACATTTTCTATCGT- NH_2-3' (SEQ ID NO:78), the primary INVADER oligonucleotide 5'-CCTTCCTTATCCTGGATCTTGGCA-3' (SEQ ID NO: 79, the ARRESTOR oligonucleotide 5'ACGATAGAAAATGTAAAGGTGCGC NH_2-3' (SEQ ID NO:80), the flap-reporter bridging oligonucleotide 5' – CGGAAGAAGCAAGTGGTGCGCCT CGTTAA-NH_2-3' (SEQ ID NO:81, and the secondary reporter-labeled probe oligonucleotide 5'-V(dG)-Cy3 CACTTGCTTCCTCC-3' (SEQ ID NO:82). Three control reaction sets were included in this experiment: 1) control reaction using a non-cascading reaction (basic INVADER, described in Example 10) to detect hMCP1 transcripts, using the 5' V(dC) probe (P3, SEQ ID NO:63) and the INVADER oligonucleotide Inv1 (SEQ ID NO:66) also used in Example 10; 2) a control reaction set designed to demonstrate the lack of cross reactivity between the oligonucleotides used for the detection of one target and the signal generating mechanism of the other target; and 3) a control set in which all primary and secondary components were present as for the multiplex reaction, but only one secondary reporter oligonucleotide was present: either for the detection of hMCP1 or hUbiquitin.

The primary reaction volumes were 10 µl and secondary reaction volumes were 15 µl. Each assay reaction comprised of 0, 1, 10 100 or 1000 amoles human ubiquitin and/or MCP1 *in vitro* transcript (SEQ ID NOS: 69 or 72, respectively) for the single and multiplex reactions, 10 pmoles each of the primary probe oligonucleotides (SEQ ID NOS:71 and 75) 5 pmoles of each primary INVADER (SEQ ID NO:66 and 79) oligonucleotides, and 20ng of CLEAVASE TthAKK enzyme in a 10 µl solution of 10 mM MOPS, pH 7.5, 100 mM KCl., 0.05% Tween, 0.05 % Nonidet NP40, 12.5mM

$MgSO_4$ Reactions were performed by dispensing 5 µl of the appropriate primary reaction mix (buffer, enzyme, $MgSO_4$, primary probe oligo and primary INVADER oligonucleotide) into the reaction vessel (low profile MJ Research, Inc. Cat.#MLL9601) and then adding 5 µl of target, or tRNA as the negative control. Samples were overlaid with colored Chill-out 14 liquid wax (MJ Research) to prevent evaporation and incubated at 60°C for 60 minutes.

After the primary reactions were completed, 5µl of the appropriate secondary reaction mixture (2.5 pmoles of appropriate flap-reporter bridging oligonucleotide [SEQ ID NOS: 76 and/or 81] 40 pmoles of ARRESTOR oligonucleotide [SEQ ID NOS:75 and/or 80] and 10 pmoles of each secondary reporter-labeled oligonucleotide [SEQ ID NOS: 77 and 82] such that the final concentration of the secondary reaction was 10 mM MOPS, pH 7.5, 0.05% Tween, 0.05 % Nonidet NP40, 20 mM $MgSO_4$) were added to each reaction and incubated at 60°C for 1hour.

The reactions were stopped by addition of 50µl of stop buffer containing 95% formamide and 10 mM EDTA. Two µl of each reaction were analyzed by both normal and reverse polarity gel electrophoresis. Samples were heated to 90°C for 1 minute immediately before electrophoresis through a 15% denaturing acrylamide gel (19:1 cross-linked) with 7 M urea, in a buffer containing 45 mM Tris-Borate (pH 8.3), 1.4 mM EDTA. An electric field of 20 watts was applied for for 1 hour in reverse orientation. The gel was scanned on the Hitachi FMBIO-100 fluorescence imager with 585-nM filter at 20 % sensitivity.

Images of the reverse polarity gel are shown in Fig. 29, panel A: basic non-cascading reaction; panel B: multiplex, cascading reaction; panel C: cascading reaction with MPC1 reporter oligo; and panel D: cascading reaction with Ubiquitin reporter oligo.

EXAMPLE 15

Detection of Human MCP1 and Ubiquitin Transcripts from Cell Lysates with a Multiplex CRE Format

The previous experiment demonstrated that the positively charged probe oligonucleotides can be used to detect *in vitro* transcripts in a cascading, invasive cleavage reaction, and that they function well in a true, multiplex reaction format. The present experiment demonstrates that the assay format described in Example 14 can be used to detect both the hMCP1 and hUbiquitin transcripts from cell lysates, and from preparations of total cellular RNA.

Cell lysates and total RNA were prepared from MG 63 cells (ATCC # CRL-1427). The cells were grown according to instructions supplied by ATCC, and by standard methods known in the art. Cells used for the lysate preparation were grown in 96 well flat bottom tissue culture plates, while cells used for the total RNA preparation were grown in 10 cm tissue culture dishes. Prior to either procedure, cells were stimulated with both human tumor necrosis factor-α (TNF-α [Calbiochem, Cat.# 654205]) and human interleukin-1β (IL-1β [Calbiochem, Cat.# 407615]). The final concentration in the induction medium was 10ng/ml for both TNF-α and for IL-1β.

Cell lysates were prepared as follows: Prior to lysis, cells were washed 2X with 200µl of phosphate buffered saline (PBS). Cells were then lysed by adding 30 µL of cell lysis buffer (20 mM Tris pH 7.5, 5 mM $MgCL_2$, 20 ng/µl tRNA, 0.5% Nonidet NP-40) and incubating at room temperature for 5 minutes. 20 µl of each lysate was transferred into a 96-well microplate (MJ Research). The plate was covered to prevent volume loss due to evaporation, and cellular nucleases were inactivated by heating the microplate at 80°C for15 minutes prior to the INVADER reaction.

Total RNA was isolated with Trizol reagent (Gibco BRL, Cat.# 15596) from stimulated and unstimulated cells following the manufacturer's protocol. Cells were grown in 10 cm plates to approximately 6-7x10^6 cells/plate and treated for 2 hours with TNF-α and IL-1β, both at 10ng/ml. The RNA was then suspended in RNAse free distilled water (USB Cat # US70783) and stored at –70°C.

In the following experiment 3 different INVADER assay formats were used. The multiplex, cascading reaction format was used to detect each analyte; the non-multiplex, (single) cascading reaction format was also used to detect each analyte; and a basic INVADER (non-cascading) reaction format was used for hMCP1detection only. All of the formats used the positively charged, labeled probes of the present invention as the detection moiety. Detection of each analyte was performed using total RNA, cell lysates and *in vitro* transcripts.

Target levels for the single and multiplex cascade reactions, as well as for the basic, non-cascading INVADER reaction were: either 0 or 1 fmole of *in vitro* transcript in 5 µl; 5 µl of cell lysate (approximately 2000 cells); or 50 ng of total RNA in 5 µl.

The multiplex, cascading reaction were prepared as described in Example 14 and included all the oligonucleotides required to detect both targets. The cascading reactions performed to detect only one target were prepared as described in Example 14, except the oligonucleotides required for the detection of only one of the targets (either hUbiquitin or hMCP1) were added, not both. The basic, non-cascading INVADER reactions were prepared as described in Example 10.

The products of the INVADER reaction were separated on reverse polarity gel electrophoresis (positively charged cleavage products) or normal polarity gel electrophoresis (full length probes) and the gels were scanned on the Hitachi FMBIO-100 fluorescence imager with 585 –nM filter at 20% sensitivity.

Images of the normal and reverse polarity gels are shown in Figures 30A and B. The normal polarity images are shown as panels below the reverse polarity panels, with the lanes showing the products of the same reactions aligned vertically. Lanes 1-4 show results with either 0 (noted by the – symbol) or 1 fmole (noted by the + symbol) of *in vitro* transcript; lanes 5-8 show results using cell lysates (approximately 2000 cells per reaction) with either no cellular stimulation (noted by the – symbol) or 4 hours of cellular stimulation (noted by the + symbol) prior to the lysate preparation; lanes 9-12 show results using approximately 50 ng per reaction total RNA with either no cellular stimulation (noted by the – symbol) or with 4 hours of cellular stimulation (noted by the + symbol) prior to the total RNA preparation. Lanes 1-3, 5-7 and 9-11 show the results

of the cascading reaction; lanes 4, 8 and 12 show the results of the basic, non-cascading reaction.

EXAMPLE 16

Detection of Positively Charged, Labeled Oligonucleotide Tags by Capillary Electrophoresis

Capillary electrophoresis (CE) is an extremely useful tool that can be used for fast and effective separation of a wide variety of molecules, including DNA oligonucleotides (Baker, D.R. (1995) Capillary Electrophoresis, Wiley Interscience Publications, New York, USA), herein incorporated by reference in its entirety. CE offers the advantages of high sensitivity, ease of use, and low cost. It provides a fast and effective method for the detection of dye-labeled tags, using, for example laser induced fluorescence. Most of the commercially available CE instruments are also capable of charge reversal electrophoresis (CRE). Therefore, it was decided to employ CRE as a method to detect the positively-charged tags generated by the invasive cleavage reactions, described and demonstrated above.

An interesting feature of the different, positively charged tags (*e.g.*, products of an INVADER assay reaction using CRE probes) is their low charge-to-mass ratio. The oligonucleotide-positive charge tags used in this study have a net charge of +1 and a mass slightly higher than that of a DNA nucleotide base. Thus, it would be extremely difficult to use the conventional CE-based DNA separation methods (such as gel-filled capillaries) because the injection times required for appropriate sample delivery would result in line broadening and poor sensitivity.

Therefore, other CE techniques, such as hydrodynamic injection and sample stacking using charged zone electrophoresis (CZE), and micellar electrokinetic capillary electrophoresis (MECC or MEKCC) (Weinberger, R. (1993) Practical capillary electrophoresis, Academic Press, San Diego, U.S.A, herein incorporated by reference in its entirety) were employed to achieve the sensitivity and resolution required for separation of the positively charged, tagged oligonucleotides.

The following examples demonstrate optimization of experimental conditions for MECC-CE based separation of the positively charged tagged oligonucleotides generated by INVADER reactions.

Optimizations of CRE Conditions: Detection of Positively Charged Oligonucleotide Tags

In order to determine the optimal conditions for running CRE experiments using capillary electrophoresis employing sample stacking and micellar electrokinetic capillary electrophoresis (MECC), a number of variables were tested. The variables were determined to have the greatest effect on the resolution and sensitivity of detection of INVADER-cleaved tag products. The CRE probes were synthesized as described in Examples 4-6. The tags are depicted top to bottom in Figure 17, and are called Tag 6, Tag 3, Tag 5, Tag 4, Tag 1 and Tag 2, respectively. The INVADER assay reactions used in these to release these tags were conducted using the oligonucleotides, target DNAs, probes and conditions described in Example 18.

Unless otherwise indicated, all experiments described below were performed on a Beckman-Coulter P/ACE MDQ capillary electrophoresis system equipped with a YAG 532 nm laser (JDS Uniphase) and a 580 ± 10 nm emission filter (Andover Corporation, Cat.#580FS10-12.5). 100 micron eCAP (Beckman-Coulter) capillary (10 cm to window) was run at 25°C with a constant separation voltage of 25 kV, using a separation buffer of 50 mM Bis-Tris borate pH 6.5. The capillary was pre-filled with 50 mM Bis-Tris borate pH 6.5 and 2% octylglucaside. The injected sample consisted of 10 nM final concentration mixture of the 6 tags in 10mM MOPS, 0.05% NP40, 0.05% Tween 20, 7.5 mM $MgCl_2$, and 10 ng/μL tRNA, and was hydrodynamically injected into the capillary using a vacuum injection of 0.5 psi from the positive electrode side of the capillary. The sample was run from the positive electrode capillary end to the negative electrode capillary end, for a distance of 10 cm to the capillary window. Data is represented as stacked traces of the raw CE chromatographs without any calculations or manipulations.

1) Effect of Sample Buffer Components on CE Resolution:

Since sample stacking relies on the conductivity and ionic strength differences between the sample buffer and the separation buffer, the effect of INVADER reaction buffer components on the efficiency of stacking was initially tested. To do this, 10 nM concentrations of each of the 6 tags were mixed in buffers containing water (A), 10 mM MOPS (B), 10mM MOPS, 0.05% NP40, and 0.05% Tween 20 (C), 10mM MOPS, 0.05% NP40, 0.05% Tween 20, and 7.5 mM $MgCl_2$ (D), 10mM MOPS, 0.05% NP40, 0.05% Tween 20, 7.5 mM $MgCl_2$, and 10 ng/μL tRNA (E), and 10mM MOPS, 0.05% NP40, 0.05% Tween 20, 7.5 mM $MgCl_2$, 10 ng/μL tRNA, and 10 ng/μL Afu FEN1 nuclease (F). Results are shown in Fig. 31.

It can be seen that the suggested minimal sample buffer components for optimal stacking and sensitivity are the presence of detergents (0.05% NP40 and Tween 20) along with 10mM MOPS. Sample in water or 50 mM MOPS did not achieve any detection suggesting that the presence of detergent is important to the method. It can also be seen that sample buffer F still allows for good resolution and detection sensitivity. Since the INVADER reactions are carried out in sample buffer F, no sample treatment (i.e. desalting or concentrating) is required prior to running CRE.

2) Injection time effects:

Effective sample stacking is highly dependent on the volume injected into the capillary (Weinberger, R. Practical capillary electrophoresis, Academic Press, San Diego, U.S.A [1993]). In this experiment, the optimal (maximum) injection volume of sample was determined. The injected sample volume that gave the best resolution was then used in subsequent experiments.

Samples were injected using a 0.5 psi vacuum for periods of 10, 20, 30, 40, and 60 seconds. Results are shown in Fig. 32 (A, B, C, D, and E, respectively). Results show that 10 to 40 seconds injection resulted in an increase in sensitivity. However, somewhere between 40 and 60 seconds a loss in resolution is apparent, suggesting that stacking is no longer optimal. Therefore a 40 second injection time was used for all subsequent experiments.

3) Effect of capillary type:

The electroendosmotic flow (EOF) of CE is very dependent on the type of capillary coating used (Weinberger, (*supra*)). Commonly used bare-fused silica capillaries have an EOF that may cause problems for certain CE applications (Baker, D.R. Capillary Electrophoresis, Wiley Interscience Publications, New York, USA [1995]). Coated capillaries are usually used as a solution to the EOF problem. There are two different types of coatings, dynamic and static. Dynamic coating is usually achieved by adding a surfactant to the capillary filling buffer. This surfactant interacts with the silanol groups of the capillary wall, minimizing the EOF. Static coating, on the other hand, is achieved by pre-treating the bare-silica capillary with a chemical that reacts with the hydroxyls of the silanol groups coating the capillary wall, thus making it neutral and eliminating the EOF. In order to determine the best coating material for optimal CRE performance several statically coated capillaries were tested. Capillaries tested were: A) 100μ eCAP DNA polyacrylamide coated capillary (Beckman-Coulter); B) 75 μ CEP coated capillary (Agilent Technologies); C) 75μ μSIL-Wax coated capillary (J&W Scientific); D) 75μ 5%T, 5%G pre-filled μPAGE capillary (J&W Scientific); E) 75 μ bare fused silica (Beckman-Coulter) (Fig. 33). Results show that capillaries with hydrophilic coatings (i.e. polyacrylamide 100 μ eCAP and 75μ CEP) yield the best separation and sensitivity. This suggests that with the appropriate coating material (dynamic or static), bare-silica can be efficiently used to resolve CRE-based INVADER assays.

4) Separation (electrode) and capillary filling buffer effects on CRE

To determine the ionic strength of the separation buffer that will yield maximum sample stacking, CRE was performed on INVADER assay tag products using 50 mM concentrations, pH 7.2 of: (A) Bis-Tris.borate, (B) Tris-borate, and (C) MOPS. For these experiments, the capillary was filled with the same buffer as the separation buffer, with the addition of 2% octylglucoside to achieve MECC conditions. Fig. 34 shows the results of the different buffers used. Optimal stacking is obtained for the buffer containing 50 mM Bis-Tris borate, pH 7.2. Next, the pH of this buffer was optimized for use in subsequent CE experiments. The buffer pHs tested were: 50 mM Bis-Tris borate buffers

of (A) pH 6.0, (B) 6.5, and (C) 7.2. Results are shown in Fig. 35. Optimal sample stacking and separation of INVADER-generated positive tags are obtained at pH 6.5.

Finally, to determine the optimal concentration of Bis-Tris.borate buffer to be used, concentrations of 25 mM (A), 50 mM (B), and 100 mM (C) - all at pH 6.5 - were tested (Fig. 36). Results indicate that the optimal concentration of Bis-Tris borate is 50 mM. The use of non-borate based buffers such as TAE, phosphate, and citrate, for example, are also contemplated.

5) Effect of Detergent on the Efficiency of MECC Separation of INVADER assay-generated Positive Tags

MECC takes advantage of interactions between the sample to be separated by CE and the hydrophilic charged ends of micelles commonly formed by detergent (Weinberger, *supra*). To determine which micelle-forming detergent would give optimal results, a number of different detergents were tested. CRT was performed using capillaries filled with 50 mM Bis-Tris borate, pH 6.5 buffer (A) without any detergent additions; (B) with 2% octylglucoside; (C) 2% NP-40; (D) 2% Tween-20; (E) 2% Triton X100; (F) 2% MEGA-9; (G) 2% Brij 35; and (H) 30 mM Sodium Cholate.

Results are shown in Fig. 37. It can be seen that optimal MECC resolution is obtained in the presence of 2% octylglucoside and that the use of NP-40, Tween-20, Triton X100, and Brij 35 result in lower resolution. The use of MEGA-9 and sodium cholate resulted in no sample detection. It is also worth noting that the presence of no detergent produced a single peak of poor resolution suggesting that sample stacking was still successful.

EXAMPLE 17

Analysis of H-phosphonate modifications by using Capillary Electrophoresis.

In this example, the products of the INVADER reactions using H-phosphonate tags described above (*e.g.*, in Example 10) were analyzed by capillary electrophoresis (CE). Compared with gel electrophoresis, capillary electrophoresis offers higher sensitivity and resolution, faster separation time, automation capabilities and the ability to use conditions that cannot be applied to a gel format, such as MECC.

Four net positively charged tags 5'-V-(Hex)-Cy3-C-3', 5'-V-(dA)-Cy3-C-3', 5'-V-(dG)-Cy3-C-3', and 5'-V-(dT)-Cy3-C-3' were generated by the invasive cleavage of the corresponding probes, as described in Example 10 (SEQ ID NOS:61-65, respectively). Briefly, 10 pmole of each probe oligo (P1, P2, P4 and P5) were cleaved in the presence of 10 fmole of human MCP1 *in vitro* transcripts for 3 hours to ensure nearly complete conversion of the probes to the cleaved products. The cleaved tags were diluted to 10 nM concentration using a solution containing 10 mM MOPS, pH 7.5, 7.5 mM $MgCl_2$, 10 ng/μL tRNA (Sigma), 0.05% Tween 20, and 0.05% Nonidet P40 to mimic the buffer conditions of INVADER reaction. The samples were separated in 60 cm eCAP DNA 100 μm diameter capillary (Beckman) under conditions of micellar electrokinetic chromatography (MECC) using a PageMDQ CE instrument (Beckman) equipped with a 532-nm laser and 580±20 nm emission filter. The capillary-filling buffer contained 50 mM bis-Tris-borate, pH 6.5 and 2% octylglucoside (Sigma) and the electrode buffers contained 50 mM bis-Tris borate, pH 6.5. The samples were injected by applying 0.5 psi vacuum to the outlet end of the capillary for 20 seconds. The tags were separated by applying 16 kV electric field, with the positive electrode connected to the inlet buffer. The separation distance from the inlet end of the capillary to the detector window was 10 cm.

Fig. 38 shows MECC profiles for the four net positively charged tags 5'-V-(HEX)-Cy3-C-3', 5'-V-(dA)-Cy3-C-3', 5'-V-(dG)-Cy3-C-3', and 5'-V-(dT)-Cy3-C-3' separated individually and as an equimolar mixture of all four molecules. Tag 5'-V-(Hex)-Cy3-C-3' produced a single band, whereas each of the tags 5'-V-(dA)-Cy3-C-3', 5'-V-(dG)-Cy3-C-3', and 5'-V-(dT)-Cy3-C-3' demonstrated two major peaks. The double-peak profiles can be explained by the presence of diastereoisomers formed during the synthesis of each of the studied tags. The stereoisomers formed by tag 5'-V-(Hex)-Cy3-C-3' are not separated under these experimental conditions. The separation of a mixture of all four tags shows only four peaks rather than expected seven peaks, suggesting that some tags or diastereoisomers have similar mobilities in these conditions.

It was observed that resolution of eCAP DNA capillaries gradually decreases after 10-20 runs, which could affect the separation of tags mixture shown in Fig. 38. When a

fresh capillary was used to analyze the same mixture of the four tags, all seven peaks were observed under the same conditions (Fig. 39).

EXAMPLE 18

Separation of net positively charged tags synthesized using phosphoramidite chemistry.

Synthesis of charge-balanced oligonucleotides can be performed using a phosphoramidite chemistry as described in Examples 4-6. In comparison with H-phosphonate chemistry used for the tags described in Examples 7 and 8, the phosphoramidite chemistry offers the advantage of using commercially available synthesizers and avoiding the introduction of centers of chirality at the phosphoramidate phosphorus atom during the synthesis. Six oligonucleotides with a general structure 5'-TagN-GCT CCC GCA GAC AC-3' (SEQ ID NO:83), where TagN denotes one of the six net positively charged modifications described in Examples 4-6, (shown top to bottom in Figure 17, and called Tag 6, Tag 3, Tag 5, Tag 4, Tag 1 and Tag 2, respectively). Each probe was cleaved in an invasive cleavage reaction with the INVADER oligonucleotide 5'-CAA AGA AAA GCT GCG TGA TGA TGA AAT CGC-3' (SEQ ID NO:84, termed 509-54-3) and the target oligonucleotide 5'-GAA GGT GTC TGC GGG AGC CGA TTT CAT CAT CAC GCA GCT TTT CTT TGA GG-3' (SEQ ID NO:85, termed 509-54-1) to generate net positively charged tags 5'-TagN-G-3'.

Each INVADER assay reaction was performed with 2 μM of one of the six probes, 0.1 μM INVADER oligonucleotide 509-54-3, 10 nM target oligonucleotide 509-54-1, and 100 ng of Ave FEN1 CLEAVASE enzyme (at 10ng/μl) in a 10 μL solution containing 10 mM MOPS, pH 7.5, 7.5 mM $MgCl_2$. The reactions were incubated at 63°C for 3 hours. Under these conditions, nearly all the probe molecules were cleaved generating approximately 2μM of each positively charged tag. The cleaved products were diluted to 10 nM concentration in a solution containing 10 mM MOPS, pH 7.5, 7.5 mM $MgCl_2$, 10 ng/μL tRNA (Sigma), 0.05% Tween 20, and 0.05% Nonidet P40 and analyzed by MECC as described in Examples 16 and 17.

Fig. 40 shows MECC profiles for each of the six net positively charged tags separated individually or as an equimolar mixture of all six molecules. Each of the tags produced a single peak, confirming the absence of chirality centers from the modifications. The MECC separation of the mixture of all six tags shows six peaks, indicating that the CE conditions described here are able to detect the differences in chemical structure of all six tags bearing net positively charged modifications. Separation demonstrating the power of the MECC assay is emphasized by the fact that modifications in two pairs of tags, Tag1/Tag2 and Tag4/Tag5, are composed of identical chemical building blocks differing only in the order of attachment, and therefore have an identical chemical composition. Nonetheless, they were easily resolved, demonstrating that the order of addition can be used as an additional variable, further extending the library of tags that can be configured from a collection of simple building blocks.

Superior resolution of MECC assay compared with gel electrophoresis is demonstrated in Fig. 41. Samples containing 0.2 pmol of 5'-Tag1-G-3' or 5'-Tag2-G-3' in 2 μL of 95% formamide, 20 mM EDTA and 0.02% methyl violet were loaded on a 100x100x2 mm slab of 20% denaturing polyacrylamide gel (crosslinked 19:1) with 7 M urea in a buffer containing 45 mM Tris-borate, pH 8.3 and 1 mM EDTA Fig, 41A) or on a 100x100x2 mm slab of 10% native polyacrylamide gel (crosslinked 19:1) in a buffer containing 50 mM bis-Tris-borate, pH 6.5 (Fig. 41B). The samples were separated by applying an electric field of 5 watts power for 30 minutes with the positive electrode connected to the top buffer reservoir (reverse orientation). The tags were visualized using FMBIO-100 fluorescence imager as described in Example 9. Fig. 41A shows that 5'-Tag1-G-3' or 5'-Tag2-G-3' have very low mobility under the conditions of the denaturing gel, precluding their identification based on this characteristic. Under the native conditions (Fig. 41B), each of the net positively charged tags was separated as two bands. There was no significant difference in the electrophoretic mobility between the two tags to distinguish them from each other.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and systems of the invention will be apparent to those skilled in the art without

departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in the relevant fields are intended to be within the scope of the following claims.